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Rodent Ultrasonic Mating Calls as a Biomarker for Oromotor Deficits in Parkinsonian Animal Model

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Rodent Ultrasonic Mating Calls as a Biomarker for Oromotor Deficits in Parkinsonian Animal Model

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2009

Dedication

This dissertation is dedicated to my parents, Kai Ma, and Hsiao-Hsia Liu, for their love and nurture that taught me to be a righteous man in this world.

Acknowledgements

I would like to first thank Dr. Schallert for his mentorship during these years. With no experience in rodent ultrasound before, he believed in me when I came to the lab. An opportunity was given with the resources I needed to accomplish these interesting studies. Countless talks and numerous ideas from him matured me as each day passed by. I gained confidence in the work that I am doing and the courage to encounter new territories without holding back. Thank you, Tim! I'd also like to thank Dr. Larry Cormack, for your co-advising and technical support that helped me carry out recordings in a noisy environment. I won't forget the numerous meetings with you on statistics! Also for my committee members, Dr. Francisco Gonzalez-Lima, Dr. Yvon Delville, and Dr. Hans Hofmann, thank you!! Your encouragement to me after giving my first talk, your arrangement of bringing in an ultrasound expert so I could pick his brain, and your willingness to listen to my story even though I'm not in the Biology field. I consider myself a blessed one of being nurtured in this enjoyable atmosphere. All of you constantly remind me how I should pass this spirit on to students I encounter in the future.

It has also been a great time working with the Schallert lab members. I specifically want to thank Drs. Michelle Ciucci and Cynthia Fox for teaching me all about speech disorders in Parkinson's patients. I'd also like to thank Jackie Kane's substantial help on surgery and animal testing, along with Marty Woodlee on helping

on the HPLC machine. I'm also grateful for the collaboration with Drs. Tobias Riede and Rod Suthers on the X-ray project. Thank you all for stimulating me to be a better researcher. And of course, how can I forget my undergrads? I would also like to thank my undergraduate team, Lynn Krug, Christine Wang, Johnny Wu, Sam Ho, Eric Chen, Sunhee Kim, Carissa Winland, Christina Sheridan, NuNue Yang, Lauren Ningcharoen, Philip Gu, Victoria Yen. All this work wouldn't be so interesting without the help you provided along. I wish you all the best in the future.

Life couldn't be easy without a strong support and a deep love for someone in a foreign land. I would like to thank Cliff & Delores Holubec for their love and treating me as a son of their own. And all the fun, laughter and tears I had with the International Student Connection gang. You are the best! I also like to thank Paul & Judith Hiller for their unlimited support and love to someone whom they've never met before. And of course, the Hyde Park Baptist Church Graduate class, your friendship and care means a lot to me.

Last, I would like to thank my parents and sisters, Kai, Hsiao-Hsia, Kuo-Lei and Kuo-Yun for their support and understanding. Thank you for letting me search my dreams in a land abroad. And of course, my wife, Pei-Lin, who trusted me in all my decisions and supported me to finish the work.

Thank you!!

Rodent Ultrasonic Mating Calls as a Biomarker for Oromotor Deficits in Parkinsonian Animal Model

Publication No._____

Teh-Sheng Ma, Ph.D.
The University of Texas at Austin, 2009

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Neurodegenerative diseases, such as Parkinson's disease (PD), likely initiate their pathologies primarily within the brain and later manifest themselves in daily behavioral functions. In patients with PD, the loss of dopaminergic neurons in the basal ganglia results in sensorimotor deficits, including tremor, bradykinesia, olfactory function loss, speech/voice loss, and eating disorders.

Although not much is known about the etiology of Parkinson's disease, extensive studies have focused on correlating different signs of motor degradation with the degree of dopaminergic neuron loss. Despite the fact that different animal models and diverse behavioral methods have been developed to further characterize limb motor function loss, the loss of fine oromotor function, which includes

eating/biting disorders and voice/speech loss, has been largely overlooked due to its intrinsic complexity as well as the lack of a precise method for quantitative description. An animal model was developed for the study of oromotor deficits in PD that utilizes the production of ultrasonic vocalization in lab rodents. Parkinsonian animals suffer the same symptoms in their vocalization compared to human PD patients: a significant drop of intensity and pitch variation. Furthermore, a newly developed biting test provided evidence that the animal's oromotor function have been compromised due to dopamine loss. Overall, these studies show that qualitative analysis of the ultrasonic vocalizations (USVs) of laboratory rats can serve as a sensitive behavioral biomarker for the detection of subtle oromotor deficits in neurodegenerative diseases.

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INTRODUCTION

Nearly 1.5 million people suffer from Parkinson's disease (PD) in the US. The major hallmark of PD is the depletion of dopamine (DA) neurons in the substantia-nigra pathway that results in symptoms that include hand tremor, gait difficulties, and akinesia. Although pharmaceutical intervention, such as Levodopa, can help relieve motor symptoms in patients, however, some fine motor skills, such as finger manipulation, cannot be reversed (Bliem et al., 2007; Moore et al., 2008). Oromotor dysfunctions, including loss or degradation of speech (dysarthria), swallowing disorder (dysphagia), and biting force loss (masticatory performance), are also prevalent among PD patients (Darley et al., 1969a; Logemann et al., 1978; Fuh et al., 1997; Miller et al., 2006). Ninety percent of individuals with PD suffer from these dysfunctions, which can markedly impact their social communication and quality of life (Edwards et al., 1991; Bird et al., 1994; Ali et al., 1996; Ho et al., 1998; Fox et al., 2002). Speech and voice loss typically occurs early in the development of the disease and is resistant to Levodopa treatment.

Although many studies and animal models have been developed to investigate the pathophysiology of the disease (Tillerson et al., 2001; Ungerstedt and Arbuthnott, 1970; Woodlee and Schallert, 2004), most attention has been focused on the restoration of gross motor function loss. There is a scarcity of animal research/literature that addresses oromotor dysfunctions in Parkinson's disease. Thus

this proposal focuses on modeling speech/voice production and mastication and assessing the effects of the loss of dopaminergic neurons in the nigro-striatal pathway.

Laboratory rodents vocalize in ultrasonic range (22-kHz ~ 80-kHz), which is outside of normal human hearing capacity (20-Hz~20-kHz). Ultrasonic vocalization has become an important behavioral measurement in rodent models of human diseases. It has been used in rat models of depression and anxiety (Mallo et al., 2007), fear conditioning (Swiergiel et al., 2007), mother-pup interactions (Branchi et al., 2001), reward systems (Burgdorf et al., 2007), and recently as a model for vocalization deficits associated with Parkinson's disease (Ciucci, Ma et al., 2007).

Rats elicit 2 types of vocalizations. They are categorized by the general frequency of the calls. One is the 50-kHz range call, the other is the 22-kHz range call. The 22-kHz call is mainly associated with aversive states, such as being defeated by a cage mate or encountering a footshock experience. The 50 kHz call is produced mainly during a positive affective state. For example, a rat re-united with its cage-mate, a mother rat re-united with pups, a male encountering a female during courtship or a rat being infused by drugs of abuse.

In human PD, the level of DA terminal loss is not symmetrical across the two hemispheres. During this hemi-Parkinson stage there is substantial dopamine depletion in the striatum opposite to the affected limbs, but sub-clinical dopamine depletion in the other hemisphere such that limb sensorimotor symptom presentation

is unilateral. It is unclear whether speech/voice deficits in hemi-Parkinson disease require at least partial loss of dopamine terminals in both hemispheres since voice production recruits muscles innervated bilaterally (Duffy, 2000). This question can be addressed by examining USVs in rats with DA depletion restricted to the nigrostriatal projections in one hemisphere.

Based on preliminary findings that the 50 kHz mating calls in laboratory rats are degraded in quality after dopamine depletion (Ciucci, Ma et al., 2007), I presented the recorded mating calls, which were sung by males to females during courtship, to estrous female subject rats. The response to these calls will help us answer several important questions: First, are the digitally recorded mating calls behaviorally relevant to the animal? Second, are they authentically captured during the recording phase? Third, will the animals display a preference for the normal mating calls versus the dopamine depleted ones? This will be a foundation for future therapeutic intervention studies on speech and voice.

Finally, swallowing and mastication deficits are also prevalent in human PD patients (Bird et al., 1994; Fuh et al., 1997). In dopamine depleted animal models, studies have reported impairments in feeding behavior due to the loss of dopamine in the terminals (Kitayama et al., 2007; Szczypka et al., 1999). No studies, however, have addressed the mastication (bite strength) loss during food ingestion. With the aid of the pasta-handling test originally designed to measure forelimb and digit function in stroke and PD animal models (Whishaw et al., 1998; Allred et al., 2008),

the acoustical information of the biting sound during food ingestion was recorded. The biting force during dry pasta eating was detected through this method using sonograms and X-ray analysis. As with USVs, unilateral DA depletion was found to degrade performance in rats.

To summarize, Parkinson's disease patients suffer from eating and speech/voice disorders as the disease progresses. These signs often occur as early symptoms. The discoveries made in the study of ultrasonic vocalization (USV) in laboratory rats could be used as a translational model for the benefit of patients who are suffering from these oromotor dysfunctions.

Chapter One: Isolate and record male ultrasonic vocalization (USV) during social interaction

INTRODUCTION

Rats communicate in a number of ways, including using ultrasonic vocalization (USV). There are a variety of situations in which rats produce USVs, including maternal localization of pups (in a mother-pup dyad), courtship behavior, exposure to drugs of abuse, sounding alarm for external danger, agonistic (promoting escape, withdrawal or dispersion), affiliation promoting conspecific contacts, phatic (maintaining contacts between individuals or cohesiveness in social groups), and alimentary/reproductive (Burgdorf et al., 2005). Improved understanding of USVs may provide insight into vocal communication and lead to studies exploring underlying neural mechanisms.

Rats produce several types of USVs that can be classified by the average frequency and complexity of the acoustic waveform. Type and complexity of the call varies depending on the age, sex, social situation, affective state, and general situation of the rat. These calls are semiotic, or have symbolic reference, and are capable of changing the behavior of the signal recipient (Blumberg et al., 1992; Burgdorf et al., 2005; Wohr and Schwarting, 2007).

Generally, the peak energy of the USV falls into 2 well-defined bands, one at approximately 22 kilohertz (kHz) and the other at approximately 50-kHz (Brudzynski and Ociepa, 1992; Brudzynski et al., 1993). The 22-kHz calls generally

occur as an alarm in response to, for example, an electrical footshock (Mallo et al., 2007), or mother-pup separation (Branchi et al., 2001). Thus, they are thought to reflect negative emotional valence (Knutson et al., 2002) and have been shown to decrease in number with anxiolytics.

Compared to the 22-kHz calls, the 50-kHz calls are more complex. They are either short, constant frequency calls (constant calls), or longer calls that are frequency modulated (FM) (Blanchard et al., 1992; Fu and Brudzynski, 1994; Wintink and Brudzynski, 2001; Brudzynski and Pniak, 2002). The 50-kHz calls can be elicited by stimulating the ventral tegmental area and its projections to the hypothalamus, nucleus accumbens and the prefrontal cortex by dopamine-mediated pathways (Burgdorf et al., 2000). Furthermore, the number of 50-kHz calls increases with electrical stimulation of these pathways, 'reward' drugs, and tickling, but is reduced with the introduction of a dopamine antagonist (Burgdorf et al., 2007). There is some evidence, however, that they can also occur in non-rewarding or perhaps even aversive contexts (Schwarting et al., 2007).

USVs have been examined in rat models of depression and anxiety (Mallo et al., 2007), aversive conditioning (Swiergiel et al., 2007), mother-pup interactions (Branchi et al., 2001), reward systems (Burgdorf et al., 2007; Ahrens et al., 2009), and parkinsonian sensorimotor function (Ciucci, Ma et al., 2007). 50-kHz vocalization is associated with sexual motivation, as males vocalize more with increasing sexual experience and when in close proximity to receptive (estrous)

females (Bialy et al., 2000; McGinnis and Vakulenko, 2003). In contrast, isolated males make fewer 50-kHz calls (in absence of conspecies) (Blanchard et al., 1991).

Unfortunately, there are several challenges to effectively using USV as a behavioral measure. One pertains to eliciting reliable amounts (numbers per session) of vocalizations as there is some inter-rat variability in the number of calls per session. However, there is relatively high intra-rat stability in repeat test conditions (Schwarting et al., 2007). Further, it seems that some rats naturally produce more calls than others and can be bred to be 'high callers' (Burgdorf et al., 2009).

Another major challenge is the difficulty in isolating the subject rat calls in the presence of other rats (i.e., the source identification problem). This is an especially important problem to solve because USVs most often occur in the context of social interaction. Previous studies have demonstrated that female rats produce USVs during copulation (White et al., 1993). We have also confirmed that receptive (in estrous) female rats vocalize immediately following the mounting and intromission. Thus, it is especially important to filter the calls of the female rats, which otherwise may lead to artificial elevation of absolute number of USVs in a session.

Finally, current methods of analyzing rat USVs, such as quantifying the number of calls produced per session, do not fully capture the complexity of the acoustic signal and the potential for exploring neural and context control. Previously, we have used USVs produced by rats as a model of the fine sensorimotor function

that may parallel key aspects of 'speech/voice' communication in humans (Ciucci, Ma et al., 2007). It is important to establish key aspects of the typical USV acoustic signal as normative data. Establishing parameters of normal USV signals for a given environmental condition may provide a context against which to evaluate changes in the USVs after brain and behavioral manipulations.

We have developed a recording chamber that isolates USV calls without interfering with social interaction, procedures for eliciting reliable 50-kHz vocalization.

METHODS

Animals

Ten male Long-Evans rats (Charles River, USA) were used in all experiments. 10 female Long-Evans rats were used for the male to female and male to female in estrous paradigms. All animals were aged 6 months at the time of testing and housed in groups of two in standard polycarbonate cages with sawdust bedding on a reversed 12:12 hour light:dark cycle. All testing occurred during the dark period of the cycle. Food and water were available *ad libitum*. Rats were handled each day prior to the experiments for 7 consecutive days. Female rats were brought into estrous through i.p. injections of 10 μg of estradiol (Sigma, USA) and 500 μg of progesterone (Sigma, USA) at 48 hrs and 4 hrs prior to the behavioral

testing, respectively. All experiments conducted were approved by the University of Texas Animal Care and Use Committee.

Unique Recording Chamber

Recent studies have shown that although female odor is sufficient to elicit USV from male mice, direct interaction between male and female further increases the number of USVs (Holy and Guo, 2005; Wang et al., 2008). This suggests that sexual intromission/ejaculation prior to experimentation reinforces the male rat's sexual motivation when seeking its mate (Lopez et al., 1999). To maximize odor cues after sexual experience, a unique recording chamber was designed. A 27cm X 27cm X 48cm Plexiglas chamber housed in a noise cancellation box was constructed (see Figure 1.1) to provide a constant odor flow from an estrous female that was previously sexually engaged with the subject male. A Plexiglas divider was placed inside the chamber to divide it into two compartments: the upper one to house the female, the lower one to house the subject male rat. Four ventilation holes (3mm in diameter) were drilled into the Plexiglas divider to allow odors to pass from the female to male rat via positive pressure from a fan located at the superior portion of the chamber (described below). Sound absorption material attached over the ventilation holes allowed odor to pass through while preventing any vocalization emitted from the female rat to penetrate. At the center of the divider, an ultrasonic microphone (CM16) with a flat frequency response (Avisoft, Germany) was mounted and pointed toward the subject male rat chamber to record the USVs.

Recorded signals were then amplified and sent to an AD/DA card (NI-6221, National Instruments, USA) and were sampled at a 200-kHz rate to prevent aliasing. Digitized signals were then saved into a .WAV format for further offline analysis. Recorded .WAV files were transferred to bioacoustics software SasLab Pro (Avisoft, Germany) for spectrogram-based parameter extraction. A 512 point Fast Fourier Transform (FFT) was used to calculate the spectrogram of the USVs.

Eliciting Ultrasonic Vocalization

We chose three different dyads to elicit calls: cagemates (familiar male to male), male to female, and male to female in estrous. Prior to the experiments with estrous females, each male rat was sexually experienced with a receptive female multiple times to ensure immediate intromission took place.

For each experiment, the male rat was placed in its home cage with each communication partner (male cagemate, novel nonestrous female, novel female in estrous) for a 2 minute familiarization phase. After 2 minutes with the communication partner, the subject male rat was placed inside the recording chamber with home cage bedding for a 2 minute habituation period. The communication partner rat was then placed in the superior portion of the chamber. Recording of USVs took place for 5 minutes.

Odor Permeation visualization

We implemented an airflow demonstration to visualize the odor permeation from the upper to lower compartments within the custom designed recording chamber. A block of dry ice that mimics the source of the female odor was placed in the upper chamber (Figure 1.2(a)). When the dry ice is exposed to positive environment pressure from the fan (Figure 1.2(b)), white cloud evaporation permeates through the apertures in the divider to the lower chamber (location of the male) (Figure 1.2(c)).

Statistical Analysis

A one-way analysis of variance (ANOVA) was performed on the absolute number of calls per session for the three different dyads (cagemate, male to female, male to estrous female). Since we initially found that males calling to estrous females were more frequent, all subsequent behavior testing and acoustic analysis was performed on data from this paradigm. A separate One-way ANOVA was performed on the absolute number of calls produced during a male to estrous female calling session for four days to examine the day-to-day variability in male to estrous female calling.

RESULTS

Recording Chamber

When both a male and female were placed in the lower portion of the chamber (location of the recording microphone) a total of 749 calls were recorded.

When both rats (n=6) were in the upper portion of the chamber (isolated from the recording microphone), zero calls were recorded, indicating that the recording microphone is isolated from the female calls from the upper portion of the chamber and effectively records only the test subject calls in the lower portion of the chamber.

Odor permeation

Results demonstrated that the dry ice evaporate (mimicking the female odor) permeates the lower chamber, transmitting female odor to the male subject rat. As shown in Figure 1.2(b), after turning on the inlet and exhaust fan located on the top and bottom of the chamber, the white cloud jets through the holes of the divider that separates the estrous female rat from the male subject rat. We overlay a quiver graph with arrows fixed at certain points in space to indicate the direction and amplitude of the airflow. Figure 1.2(c), mimics the situation when the estrous female odor is fully pervaded in the lower chamber where the subject male rat is resided. This setup allows the estrous odor to permeate but prevents female USV signal transmission. Thus, the odor of the female is likely an adequate stimulus for the male subject rat.

Dyads for eliciting calls reliable calling

Results of the ANOVA revealed that the social paradigm that produced the greatest numbers of calls per session was a sexually experienced male calling to a familiar estrous female ($F(2, 23) = 7.623$, $p < 0.01$) (Figure 1.3). The fewest amount of vocalizations produced per session was the familiar male to male paradigm (mean

= 48.80, SE = 19.30). The next greatest number of calls was male to novel nonestrous female (mean = 137.5, SE = 49.72), followed by male to novel estrous female (mean = 259.6, SE = 50.49).

Day to day Variability

The absolute number of calls observed in a 5 minute testing session in the male to estrous female showed some day to day variability (see Figure 1.4), but this was not significant ($F(3, 27) = 3.18339$, $p > 0.05$).

DISCUSSION

Communication, particularly vocalization, is a vital function of survival and well-being. Deficits in vocalization are often the first signs of neurological disease, yet voice and speech sensorimotor functions are often overlooked in studying the nervous system in both basic and clinical sciences. However, recent work in non-human mammalian vocalizations have contributed to our understanding of both normal and abnormal physiology (Ciucci, Ma et al., 2007).

In developing a rat vocalization model, several challenges arose. The primary challenge was in eliciting consistent vocalizations from the male rats. Previous studies have shown that rats produce the greatest amount of vocalizations during social interaction, specifically sexually-experienced male rats calling to female rats (McGinnis and Vakulenko, 2003). We examined absolute number of calls per session in three conditions: male to male calls (cagemates), male to novel nonestrous

female calls, and male to female in estrous calls. The greatest number of vocalizations occurred when a male rat called to a female estrous rat.

The second major challenge was in isolating male rat calls from female calls for analysis. Examining USVs within a social context is behaviorally relevant. However, in social situations, it is difficult to isolate the target source of vocalization (isolate which rat is calling). Studies indicate that USV is not limited to male rodents, but also occurs in females during resident-intruder paradigm or when food cues from conspecifics are present (Moles and D'amato, 2000; D'Amato and Moles, 2001). Thus, it is critically important to isolate the calls of a test subject, especially when comparing acoustic properties of the vocalizations during experimental manipulation. Although USV can be evoked solely through odor stimulation from male mice (Holy and Guo, 2005), a recent publication has confirmed direct sexual contact between male and female mice increases the number of USVs produced by males several-fold, while not increasing the number produced by females (Wang et al., 2008). However, in our experience, communication during sexual behavior appears more complex in laboratory rats as compared to mice, as female USV also plays an important role in the courtship behavior (Thomas and Barfield, 1985; White and Barfield, 1987). To eliminate the source of female USV production without devocalizing the females or substantially modifying male to female interactions, we designed a recording chamber that allowed female odors to permeate to the male portion of the chamber and recorded only male vocalizations. Thus it is capable of

sustaining social interaction while isolating the subject male's USV from the communication partner's USV. This was verified with acoustic data and an odor permeation simulation.

Most studies have focused on using the absolute numbers of calls as an index of behavior. While this is useful in some models, we found that the hemi-parkinsonian rat vocalization deficit does not manifest in the absolute number of calls produced, but rather in degradation of the acoustic signal (Ciucci, Ma et al., 2007). However, spectral acoustic analysis allows for a variety of measures in regard to duration, frequency and intensity measures. Based on these categories of calls and the salient acoustic parameters that define them, it is possible to then examine *how* the acoustic properties of the USVs are altered in models of sensorimotor or cognitive deficits.

These methods should be useful in the study of vocalization in animal models of neurodegenerative diseases and in psychopharmacology.

FIGURES

Figure 1.1 Custom built acoustic recording chamber to record the male rat's ultrasonic vocalization.

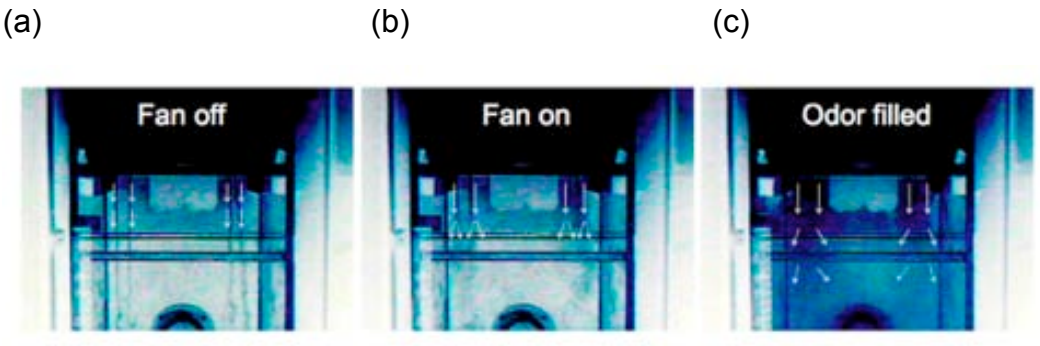

Figure 1.2 Odor permeation experiment simulating the flow of female rat odor from the top chamber down to the male recording chamber. (a) Simulates female odor filled in the upper chamber with circulating fan off. White arrow depicts streaks of odor passively passing through the odor 4 odor holes. (b) After turning on the circulating fan, simulated female odor actively passes through the odor holes resulting a turbulent flow condition. (c) As the simulated female odor gets pulled through more by the fan, the lower recording chamber is filled with the simulated odor (dark area with white arrows).

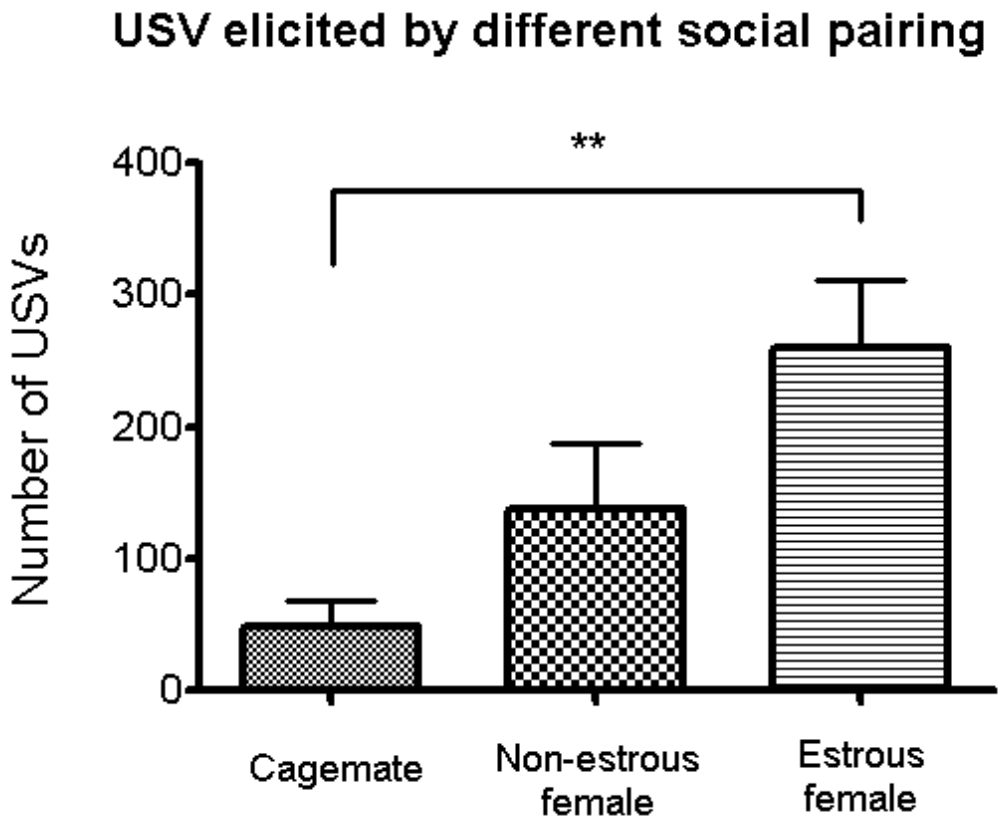

Figure 1.3 Different social pairing induced ultrasonic vocalization in male laboratory rats. Error bars represent Standard Error of the Mean (SEM).

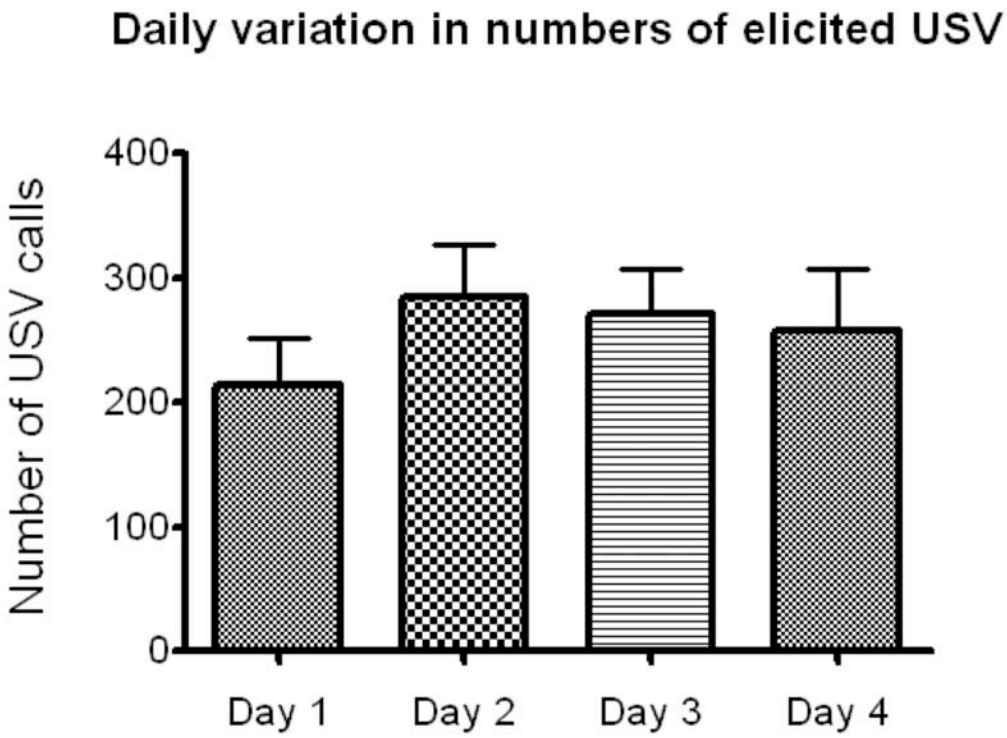

Figure 1.4 Daily variation of elicited USV in male laboratory rats. A total of 4 days were monitored. Error bars represent Standard Error of the Mean (SEM).

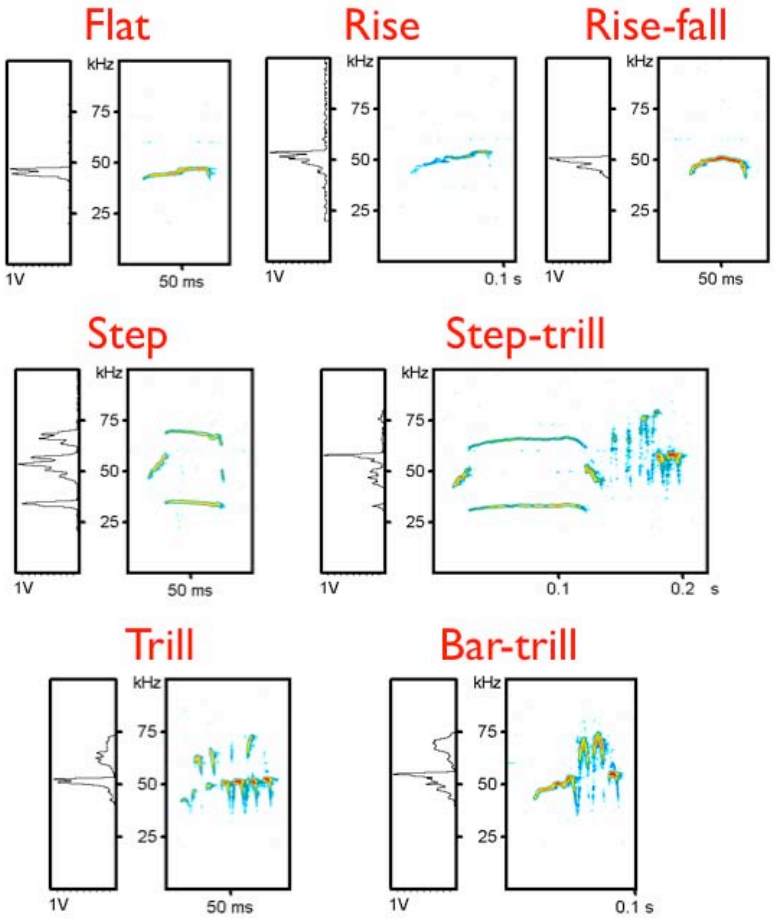

Figure 1.5 Seven call types of the 50-kHz vocalization of laboratory male rats during sexual context.

Chapter Two: Qualitative changes in rat USV after dopamine depletion

INTRODUCTION

Up to 89% of individuals with Parkinson disease (PD) suffer from disordered speech and voice that significantly impacts their quality of life (Logemann et al., 1978; Ho et al., 1998; Fox et al., 2002). Speech/voice disorders (i.e., dysarthria) are often common in the early stages of the disease (Darley et al., 1969a; Darley et al., 1969b; Logemann et al., 1978; Ho et al., 1998; Fox et al., 2002). However, more attention has been given to the sensorimotor deficits that occur in the limbs, which are more noticeable to patients compared to the subtle changes in speech and voice. The most salient signs of speech/voice deficits in PD patients are: decreased vocal loudness, decreased frequency variability (monotone), breathiness, hoarse voice quality, and imprecise articulation (Darley et al., 1969a; Darley et al., 1969b; Logemann et al., 1978). In spite of these deficits, the diagnosis of voice/speech disorder in PD is commonly overlooked. Furthermore, therapeutic benefits of levodopa have contrasting effect on the limbs compared to voice/speech in PD. Patients recover favorably well in motor functions when on levodopa treatment (Ramig et al., 1995; Ramig et al., 1996; Ramig et al., 2001), but show no significant progress in the recovery of voice/speech disorder (Schulz, 2002; Krack et al., 2003; Pinto et al., 2004). This brings forth an impetus for developing an animal model to study speech/voice disorder in PD.

Rodents have become a common animal model for life science research. Although rodent models provide a useful tool in studying sensorimotor deficits associated with PD (Schallert et al., 2000; Tillerson et al., 2001; Cenci et al., 2002; Fleming et al., 2005; Meredith and Kang, 2006), to date there are no rodent models to examine qualitative changes in the ultrasonic vocalization (USV) acoustic signal. As mentioned in Chapter 2, rats produce several types of USVs that can be classified by frequency and complexity of the waveform. Generally, the peak energy of the calls falls into two main categories: 22-kHz or 50-kHz frequency ranges (Brudzynski and Ociepa, 1992; Brudzynski et al., 1993). The 22-kHz calls generally occur as an alarm. This type of call is long in duration (up to seconds) with no pitch (frequency) variation. Compared to the 22-kHz calls, the 50-kHz calls are more complex. They include constant frequency calls (flat calls) or frequency modulated (FM) calls (Blanchard et al., 1992; Fu and Brudzynski, 1994; Wintink and Brudzynski, 2001; Brudzynski and Pniak, 2002). Expression of 50-kHz vocalization is correlated with sexual motivation, as males vocalize more with increasing sexual experience and with receptive (estrous) females (Bialy et al., 2000). McGinnis and Vakulenko have found that when the males were allowed to ejaculate the number of vocalizations was significantly higher (McGinnis and Vakulenko, 2003). Their results suggest that environmental stimuli play an important role in enhancing or increasing the number of 50-kHz calls per recording session.

Speech and voice deficits occur early in PD. There is evidence that

degeneration of neurons occurs in lower brainstem structures, near the dorsal motor nucleus of the vagus (Braak et al., 2003), prior to detectable degeneration of nigrostriatal neurons, and it progresses rostrally. It has been argued that voice and speech deficits might be linked to the loss of non-DA neurons in the brain stem, and that the loss of nigral DA neurons may be unrelated to these deficits. Alternatively, it is possible that during this early stage dopamine terminal loss can also lead to degradation of at least some vocal function, as it does in other fine sensorimotor behaviors. Even if this is so, an addition question is whether the loss of DA neurons must be bilateral. In the course of hemi-Parkinson stage there is substantial, but subclinical dopamine depletion in the less depleted hemisphere so that limb sensorimotor symptom presentation is unilateral. It is unclear whether speech deficits in hemi-Parkinson disease require at least partial loss of dopamine terminals in both hemispheres. In an animal model, we began to address these issues by targeting dopamine-specific neurons, and only in one hemisphere unilateral infusion of the dopamine neurotoxin 6-OHDA was used to kill DA neurons in one hemisphere. The primary outcome measure was ultrasonic vocalization. An additional dopamine depletion model was employed to test bilateral effects. Haloperidol was chosen based on its ability to block D1 and to a greater extent D2 dopamine receptors (Cohen and Lipinski, 1986) at a dose that did not affect movement initiation.

This is the first study to document dopamine deficiency linked qualitative changes in acoustic parameters of the 50-kHz USV in rats. Although a high dose of a

dopamine antagonist can reduce the absolute number of 50-kHz calls (Burgdorf et al., 2007), other changes in frequency modulation of these calls have not been examined after doses that do not substantially reduce the number of 50-kHz USVs or after unilateral disruption of dopamine. In the present study we created a paradigm to examine the effects of unilateral 6-OHDA induced degeneration of dopamine neurons and low doses of the dopamine antagonist haloperidol on rat USV. It was hypothesized that the dopamine-altered conditions would cause a degradation of the acoustic signal.

METHODS

Animals

A total of 16 male and 10 female Long-Evans rats were used in this study (Charles River). The female rats were used only to provide sexual experience and odor cues to elicit mate calling in all of the male rats. Four of the 10 males failed to mount and/or vocalize within baseline parameters and were therefore excluded from further analysis. The six male rats received both haloperidol and saline control on different days. Six additional male rats received unilateral nigrostriatal infusions of 6-OHDA (2 weeks before being tested for mounting latency and USV quality). The age of the rats at the time of testing ranged from 9-15 months, which reflects approximately the middle months of the rat lifespan (average lifespan is about 2.5 years). Animals were housed 2 per cage in standard polycarbonate cages with

sawdust bedding, and food and water were provided ad libitum. Lights were maintained on a reverse 12:12h light: dark cycle, with all behavioral procedures occurring during the dark period of the cycle. Rats were handled for 7 days prior to behavioral testing, and habituated to the recording environment for 3 days prior to USV recording sessions. All procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

Overview of Testing

All male rats were sexually experienced with female rats prior to testing. Testing included mounting latency and USV recording. Testing was performed one hour after control injections or haloperidol and 5 weeks after surgery for the 6-OHDA rats. Striatal dopamine content was estimated by behavioral measures in the 6-OHDA animals (which were carried out after USV recording), and with high pressure liquid chromatography after animal sacrifice. One 6-OHDA rat had only 12 % dopamine depletion and no behavioral asymmetries; therefore, its USV data were not included in the analysis (for 6-OHDA group, final n=5).

Dopaminergic Neurotoxin and Antagonist

Haloperidol

Rats in the haloperidol condition were injected with 0.1 mg/kg haloperidol (i.p.). Dose-response curves in our lab ranging from 0.05 - 0.5 mg/kg indicated that

at 0.1 mg/kg, most rats continued to mount and vocalize within normal limits of baseline rates, with little or undetectable hypokinesia in an open field.

6-OHDA

Moderate to severe degeneration of presynaptic striatal neurons is typically induced by unilateral infusion of 7 ug of 6-OHDA into the medial forebrain bundle (Fulceri et al., 2006; Marshall, 1979; Tillerson et al., 2001; Ungerstedt & Abuthnott, 1970). 6-OHDA treated rats were anesthetized with i.p. injections of 90 mg/kg ketamine and 10 mg/kg xylazine, and placed in a stereotaxic frame. All rats received unilateral infusions of 7 μg 6-OHDA hydrobromide (free base weight) dissolved in 3 μl artificial cerebrospinal fluid (composition: NaCl, KCl, CaCl2, MgCl2*6H20) containing 0.05% (w/v) ascorbic acid. Infusion coordinates were measured from bregma (-3.3 AP; ±1.7 ML; -8.0 DV from dural surface), and infusions were delivered at a rate of .3 μl/min for 10 minutes. Infusions were into the nigrostriatal projections in the hemisphere contralateral to the preferred forelimb, determined from baseline scores on a forelimb-use asymmetry test. Following surgery, animals were placed in a humidified incubator to prevent hypothermia and dehydration, and upon waking were returned to their home cages.

Validation of Striatal Dopamine Degeneration

To estimate the degree of 6-OHDA induced degeneration, two behavioral tests were administered: forelimb-use asymmetry and apomorphine-induced rotation.

At the completion of all experiments, striatal dopamine content was analyzed with high pressure liquid chromatography with electrochemical detection (HPLC-EC).

Behavioral Tests

Rats treated with 6-OHDA were tested for forelimb-use asymmetry on post-surgery days 7, 14, and 55 (Schallert & Tillerson, 2000; Schallert & Woodlee, 2005; Schallert et al., 2000) by placing them in an upright acrylic cylinder (diameter 20 cm) to encourage rearing and exploratory movements with the forepaws. The number of wall contacts made by either forelimb or by both forelimbs simultaneously was recorded. The percentage of contacts made by the non-impaired forelimb relative to the total number of contacts was calculated using the formula: (ipsilateral limb contacts + both (simultaneous or rapidly alternating) limb contacts)/ total number of contacts (limited to 20 per test day to prevent habituation). Scores significantly above 50% indicate a greater reliance on the ipsilateral limb for voluntary movement and have been well correlated with the degree of nigrostriatal dopamine depletion induced by 6-OHDA lesions (Schallert et al., 2000; Ariano et al., 2005).

Apomorphine-induced rotational behavior was also examined. At 4 weeks post-surgery, animals were given 0.5 mg/kg apomorphine (s.c.), and the net number of contralateral quarter turns made during a 5 min trial was recorded (25 min post injection) (Herrera-Marschitz, Casas, & Ungerstedt, 1988).

HPLC

Rats were deeply anesthetized with halothane and decapitated. Brains were removed and the dorsal halves of the left and right striata were each dissected out over ice. Striata were then accurately weighed before being sonicated in 40 µl of cold mobile phase (see below) per mg of tissue. The sonicated suspension was then centrifuged at 13,500 RPM for 15 min at 4°C and the supernatant was removed for analysis by high pressure liquid chromatography with electrochemical detection (HPLC-EC). Ten microliters of each sample were injected onto a BDS Hypersil C18 100 x 3 mm column (held at 40°C, and preceded by a 10 mm C18 guard column). The mobile phase consisted of a 15 mM sodium acetate / 20 mM citric acid buffer (pH 3.7) containing 70 µM disodium EDTA, 0.04% (w/v) sodium 1-heptanesulfonate, and 10% (v/v) methanol. This mobile phase was pumped through the system at 0.9 ml/min. A guard cell set at +450 mV was placed between the pump and sample injector to reduce electroactive impurities in the mobile phase. Eluates from the column were analyzed by an ESA Model 5011A analytical cell coupled to a Coulochem II controller. The first electrode was set at a potential of -100 mV and the second was maintained at +400 mV. The heights and retention times of current peaks recorded from the detecting (second) electrode were compared against those produced by a series of standards (obtained from Sigma) of known concentration to ultimately determine the striatal content of dopamine in terms of µg of substance per g of wet tissue weight. When samples fell below the limit of detection, a value

halfway between zero and the detection limit was recorded for that sample. Lesion severity is expressed as percent dopamine loss in the lesioned hemisphere, relative to the intact hemisphere.

USV recording apparatus

A limitation in most USV studies has been the inability to isolate male rat calls in the presence of females. Thus, the recording environment described in Chapter 1 was applied. An ultrasonic microphone with high directional properties for recording 50-kHZ USVs (CM16, Avisoft, Germany) with a flat frequency response of up to 150-kHz and a working frequency response range of 10-180-kHz, was attached to a panel. The panel was then placed in the top center of a 10 x 10 x 12 cm sound-isolated Plexiglas chamber to divide the space into upper and lower compartments. The subject male rat was placed in the bottom area with the microphone. During the control and experimental recordings, the high frequency gain was kept at the same level. The female estrous rat was placed on top of the divider. Four 3mm holes were drilled into the panel to allow odor to pass to the male chamber. The diameter of the holes was smaller than the 50 kHz USV wavelength to sufficiently attenuate female USVs. Additionally, inlet and exhaust fan were installed to create positive pressure within the chamber to enable the female's odor to pass down to the lower chamber.

Behavioral Procedures

To maximize the number of USVs available for analysis, all males in this study were sexually experienced with estrous females prior to recording. Females were brought into estrous through s.c. injections of 10 μg estradiol benzoate and 500 μg progesterone, administered 48 hrs and 4 hrs prior to sexual encounters, respectively.

Prior to USV recording sessions, a receptive female was placed in the male's homecage, and the male was allowed to mount twice without ejaculation before the female was removed. Mount latency was recorded to ensure that the unilateral dopamine depleted and haloperidol treated rats displayed normal appetitive/motivational behaviors. Rats that failed to mount twice within 5 minutes were excluded from the study. The male was then placed in the lower compartment of the recording chamber with homecage bedding and the female was placed in the upper compartment. USVs were recorded from the male for 5 minutes. The chamber was cleaned with ethanol between trials to remove the odors of male rats, although we have not found that latent male odors in this apparatus per se elicit USVs.

Data Recording and Analysis

Video recordings with a Panasonic PV-DV800 Infrared Camcorder were made with each session to ensure that behavior, rearing, and distance from the microphone were similar among all rats. However, mouth to microphone distance was not perfectly controlled. As such, selection of calls for analysis (discussed

below) was designed to minimize the variability that may occur in analyzing intensity (loudness) as a result of slightly variable distances from the microphone.

USV recordings were collected on a computer and transferred to an external hard drive for storage and analysis. Analog recordings were digitized through a D/A card (National Instruments, USA) at 200-kHz sampling rate with 16 bit resolution. Recorded USVs were analyzed with Saslab Pro (Avisoft, Germany). Sonograms were generated under a 512 FFT-length and 75% overlap frame setup. A 300 s duration of vocalization recording was inspected after bypassing the initial 30 s of data collection to eliminate variability as the rats initially explored the chamber. Individual calls were then separated into single WAV audio file format for further parametric analysis. Calls were selected based on the quality of the acoustic signal (free from extraneous noise, sufficient energy in the signal), and all effort was applied to attempt to control for mouth to microphone distance. For the remaining dependent variables, 10% of each type of call and a minimum of 10 calls per animal were analyzed. However, not all rats made every type of call and not all calls were free from noise. Typically, the rats reared upwards toward the microphone while vocalizing. Because rats are different sizes and behave differently, the distance from their mouths to the microphone was not completely controlled. To offset this limitation, the 'best' calls were selected, meaning the calls that were the loudest (highest intensity on the spectrogram) and clearest upon visual inspection. This sampling technique was standard among all three groups and models a human speech

assessment method of analyzing the samples that have the same fundamental frequency and intensity level. Further, we analyzed the percent of time spent rearing to ensure that all three groups were behaving similarly.

In this particular social paradigm, rats make three types of calls: Simple, Frequency Modulated (FM), and Harmonic (See Figure 2.1). Simple calls have a constant frequency, without frequency modulation. The number of calls made in each call category was counted. Several dependent variables for the acoustic signal were operationally defined and selected for offline spectral analysis:(1) Duration: offset of the signal minus the onset in seconds; (2) Bandwidth: maximum minus minimum frequency in Hertz (Hz); (3) Maximum frequency: highest frequency in kHz observed in a call of the same type, and (4) Maximum intensity: maximum intensity measured in decibels (dB). Note: Intensity in dB is measured against a reference point that is internal to the microphone. This reference is a negative value. Thus, the intensity measures are reflected in negative values and the less negative value in dB reflects a louder vocalization.

Statistical Analysis

Percentage of call type was analyzed with a Kruskal-Wallis One-Way Analysis of Variance (ANOVA) and post-hoc testing was performed with a Least-Squared Difference (LSD). Duration, Bandwidth, Maximum Frequency, and Intensity were analyzed with a 3x2 Mixed ANOVA with dopamine condition (control, haloperidol, 6-OHDA) and call type (Simple, FM,) as the factors; Control

and haloperidol groups were within subject. Post-hoc testing was performed with a Tukey HSD. The Harmonic type calls were not included in this analysis as they did not comply with the selection criteria, especially due to the low intensity level. The Harmonic calls are also infrequently produced. A Bonferroni adjustment was made to the alpha level to control for the number of dependent variables in the parametric ANOVA (.05/4=.0125).

RESULTS

Percent Call Type

The type of call produced (Simple, FM, or Harmonic; Figures 2.1 and 2.2) was expressed in percentage of total calls and compared among control, haloperidol and 6-OHDA animals using a Kruskal-Wallis One-Way ANOVA. The absolute number of calls was not different between the control, haloperidol, and 6-OHDA groups. The percent of Simple calls was significantly greater than Harmonic in all groups (Control: $t=4.587$; $p<0.01$ Haloperidol: $t=7.252$; $p<0.01$; 6-OHDA: $t=3.541$; $p<0.05$). Likewise, the percent of FM calls was significantly greater than harmonic for all groups (Control: $t=3.331$; $p<0.05$, Haloperidol: $t=4.246$; $p<0.01$, 6-OHDA: $t=4.207$; $p<0.05$), meaning that rats produce more Simple and FM calls than Harmonic calls regardless of dopamine depletion. The relative amount of call types produced revealed that the number of FM calls was greatest for the control ($t=3.331$;

$p<0.05$) and haloperidol groups ($t=4.264$; $p<0.01$). FM represented the highest percentage of call type for the control and haloperidol animals, but not for the 6-OHDA animals. For the 6-OHDA animals, Simple was the most frequently observed versus other types of calls ($F(2, 16)=3.876$; $p=0.0457$).

Duration

There were no significant main effects for duration (Figure 2.3) ($F(2, 34)=2.485$, $p=.102$) or interactions for duration, although Call Type approached significance ($F(1, 33)=5.19$, $p=.031$), in that the FM calls had a longer duration than the Simple calls. Duration was not an acoustic parameter affected by dopamine depletion.

Bandwidth

There were significant main effects for bandwidth (Figure 2.4) for DA condition ($F(2, 34)=64.671$, $p<.0001$) and Call Type ($F(1, 33)=8.079$, $p=.002$) and no interactions. Post-hoc tests revealed that Bandwidth was significantly greater in the control condition versus the haloperidol ($p=.004$) and 6-OHDA ($p=.006$) conditions. The DA-altered conditions were not significant by different from each other ($p=.998$).

Maximum Frequency

There was a significant main effect for maximum frequency (Figure 2.5) for Call Type ($F(1,33)=46.786$, $p<.001$). DA condition approached significance

(F(2,34)=3.763, p=.036) and there were no significant interactions. Overall, the control rats had a higher maximum frequency than the DA-altered rats for both the Simple and FM calls, but this was not statistically significant (p=.034) given our conservative p-values.

Maximum Intensity

There were significant main effects for maximum intensity (Figure 2.6) for DA condition (F(2, 34)=6.140, p=.006) and Call Type (F(1, 33)=8.721, p=.006) and no significant interactions. Post-hoc tests revealed that intensity was significantly higher in the control condition versus the 6-OHDA condition (p=.008) and approached significance versus the haloperidol condition (p=.031). The DA-altered conditions were not statistically significant from each other (p=.766).

Striatal Dopamine Degeneration

The range of asymmetry scores in the test of forelimb use during exploration of the walls of a cylindrical enclosure for the 6-OHDA rats was 75 to 90% and, in the apomorphine rotation test, an average of 300$\pm$ 54 contra turns in 5/6 rats. These data suggested that in these 5 rats there was extensive depletion, which was confirmed by HPLC (below). The one rat that did not show motor asymmetry in either test had only a minimal (12%) depletion as indicated by HPLC analysis of DA content; therefore its data were excluded from analysis. Levels of dopamine (DA) in the dorsal striata of 6-OHDA-lesioned rats were assayed via HPLC-ED, as described

above. DA levels in the DA-depleted striatum of all five animals were below the limit of detection for the assay (<194 ng DA/g wet tissue weight), indicating successful severe unilateral DA depletion. Unlesioned striata contained 9029 ± 1995 ng DA/g (mean ± SEM). High variability on the unlesioned side was due to one outlier who was observed to have a low DA level of only 1325 ng/g in the unlesioned striatum, for unknown reasons. Exclusion of this animal yielded a mean unlesioned-side DA content of 10955 ± 671 ng/g. Assuming a dopamine level halfway between 0 and the limit of detection (i.e., 97 ng/g) for the lesioned striata, the mean percentage depletion was calculated as 97.8% ± 1.3% DA loss compared to the intact side. The animal with a DA depletion on unlesioned side still demonstrated forelimb asymmetry, as the lesioned side was DA depleted to a greater degree.

DISCUSSION

Sexually experienced male rats were paired with estrous female rats and their USVs were recorded in a sound-treated chamber that isolated male rat calls. Complex 50-kHz ultrasonic calls were substantially degraded in a unilateral hemi-Parkinson rat model and after the DA antagonist antipsychotic drug, haloperidol. Adequate DA synaptic activity, therefore, may be required for normal 50-kHz vocalization. These data may be relevant to discussion of the role of nigrostriatal DA neurons versus brainstem non-DA neurons on phonation deficits in the early stages of Parkinson's disease. It remains possible that midbrain DA projections at least

modulate this complex oromotor function. Call intensity and bandwidth in both simple and frequency modulated calls of the 50-kHZ USV type were significantly reduced. Duration and maximum frequency were reduced but the extents of reduction were not statistically significant. The total number of calls in the 6-OHDA and haloperidol treated rats was not reduced. The average degree of dopamine depletion in the lesioned hemisphere was 97%, which is considered a severe depletion. The behavioral deficits were slightly less severe at the times of assessment. Relative to control, there was about an 80% decline in use of the contralateral forelimb for exploration in the rats with severe unilateral dopamine depletion. In these same rats, 50-kHz call bandwidth and intensity were reduced by about 40% and 23%, respectively. Given that the ipsilateral forelimb was not impaired, the overall decline in bi-forelimb function could be considered roughly similar to the reduction in USV bandwidth and somewhat more severe than the reduction in USV intensity. Future studies should include an exploration of the relationship between degree of dopamine depletion and acoustic impairment across a wide range of depletion, as well as the social implications of these deficits on mating behavior.

Previous research has shown that *bilateral* 6-OHDA-induced degeneration of dopamine neurons can diminish the number of calls (Burgdorf et al., 2007). Others have shown that haloperidol injected into the nucleus accumbens caused a significant increase in the number of 50-kHz calls, although this was related to injection site

(Thompson et al., 2006). Additionally, data showed that haloperidol did not block the animal's ability to produce 50-kHz calls, but may have reversed glutamatergic effect to the level of spontaneously emitted calls (Wintink and Brudzynski, 2001). However, examining only the number of calls that occur in a situation may overlook important data concerning the degree of signal degradation in the callings. Although the number of calls per session was diminished in some of the animals following haloperidol, all were within the range of number of calls observed in previous habituation periods. The data do not appear to reflect simply a decrease in the salience of the female stimulus. The time to mount an estrous female was not statistically different between control and dopamine-altered conditions, although the mounting times for the control condition were more reliable and less variable. Sensorimotor feedback impairment, incentive salience, and fatigue influences cannot be ruled out as contributing factors, but seem insufficient to account for the reduced bandwidth and diminished intensity of the acoustic signal while maintaining the absolute number of calls. In another pilot study, Levodopa was tested and did not display therapeutic effects on the quality of the vocalization, which is in line with human PD patients. That is, voice and speech deficits do not respond substantially to Levodopa at a stage when other motor signs do (Pinto et al., 2004; Schulz, 2002, Curr Med Chem).

The mechanism of USV production in rats is poorly understood. Generally, respiratory, phonatory/laryngeal, and possibly articulatory subsystems of

communication are utilized. Constant USVs are produced by a whistling mechanism by approximation and fixation of the vocal folds (Roberts, 1975). FM calls are possibly produced by a similar whistle mechanism and the modulation is thought to be produced by articulation at the level of the larynx. However, the respiratory and upstream (tongue) subsystems of the vocal tract are likely candidates for modulation as well. Understanding the mechanism of modulation will facilitate understanding the complexities of the communicative motor system and how it breaks down with neurological disease.

This is the first study to document a qualitative change in the acoustic signal of frequency-modulated 50-kHz calls as a result of interfering with dopamine synaptic transmission in rats. These findings may be useful since speech/voice deficits are prevalent in individuals with PD and the neural mechanisms underlying speech/voice disorders in PD are unclear (Estenne et al., 1984; Ackermann and Ziegler, 1991; Ackermann et al., 1997; Fox et al., 2002; Trail et al., 2005). Bradykinesia and hypokinesia associated with PD are the most frequently implicated causes of the speech-system disorders (Fox et al., 2002; Trail et al., 2005; Sapir et al., 2006) and are likely linked with dopamine depletion. However, non-dopaminergic events, such as the degeneration of non-dopaminergic neurons, cannot be ruled out (Braak et al., 2004). Speech/voice impairment in PD presumably emerges as a result of faulty sensorimotor processing, which could lead to a reduced gain in the motor command for selecting and reinforcing movement amplitude

(Berardelli et al., 2001; Turner et al., 2003; Desmurget et al., 2004). These subtle changes in amplitude gain commands may interfere with shifting sets (high/low) and degrade the ability of the system to produce complex output (e.g. frequency modulation).

Based on our findings from rats treated unilaterally with 6- OHDA or with low-dose haloperidol, alteration of dopamine synaptic transmission appears to contribute to the degradation of the acoustic signal in rat 50-kHz USVs. Examining qualitative acoustic changes in rat USV may be useful for systematically investigating potential pharmacological, surgical, and behavioral treatments in dopamine-altered rats.

FIGURES

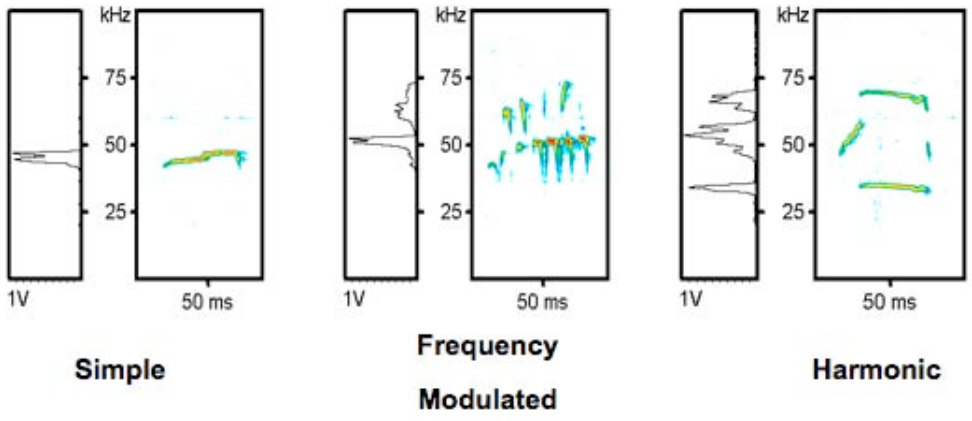

Figure 2.1 Representative Sonograms of Simple, Frequency Modulated (FM), and Harmonic calls from a rat in the Control Condition.

Left-hand boxes represent the amplitude spectrum of the call expressed in volts. Right handed boxes are the sonogram with time in milliseconds represented on the X-axis and frequency in kilohertz on the Y-axis. Relative amplitude is represented by color.

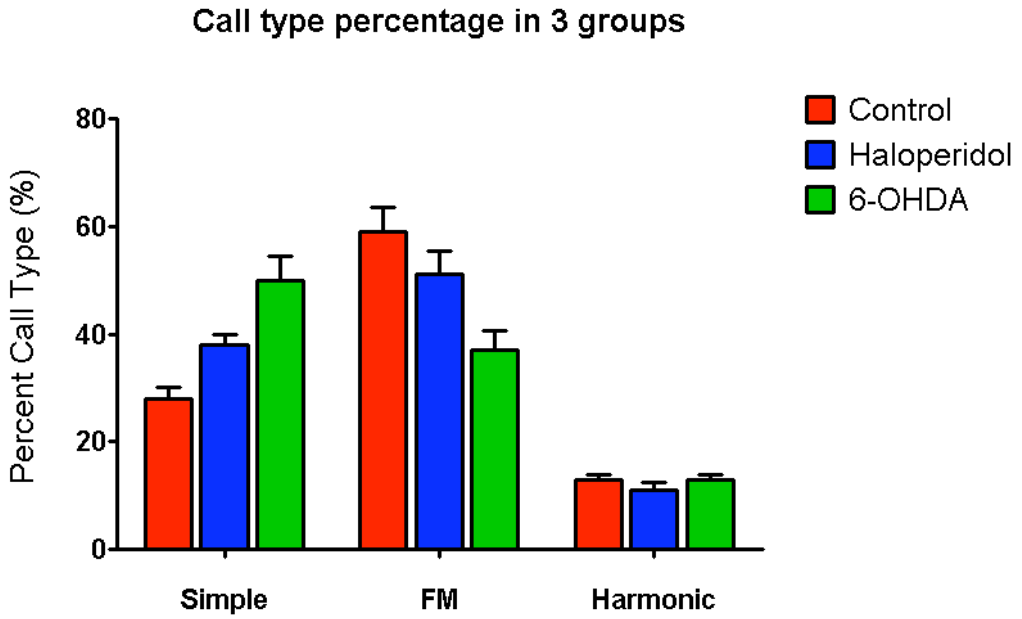

Figure 2.2 The percent of each call type (Simple, FM, Harmonic) produced in the Control, Haloperidol, and 6-OHDA conditions ($p < .01$).

Overall, the most common Call Type produced is FM, followed by Simple and the least common is Harmonic. For the Control condition, the most common type of call is the FM call. In the 6-OHDA condition, more Simple calls were produced than FM or Harmonic.

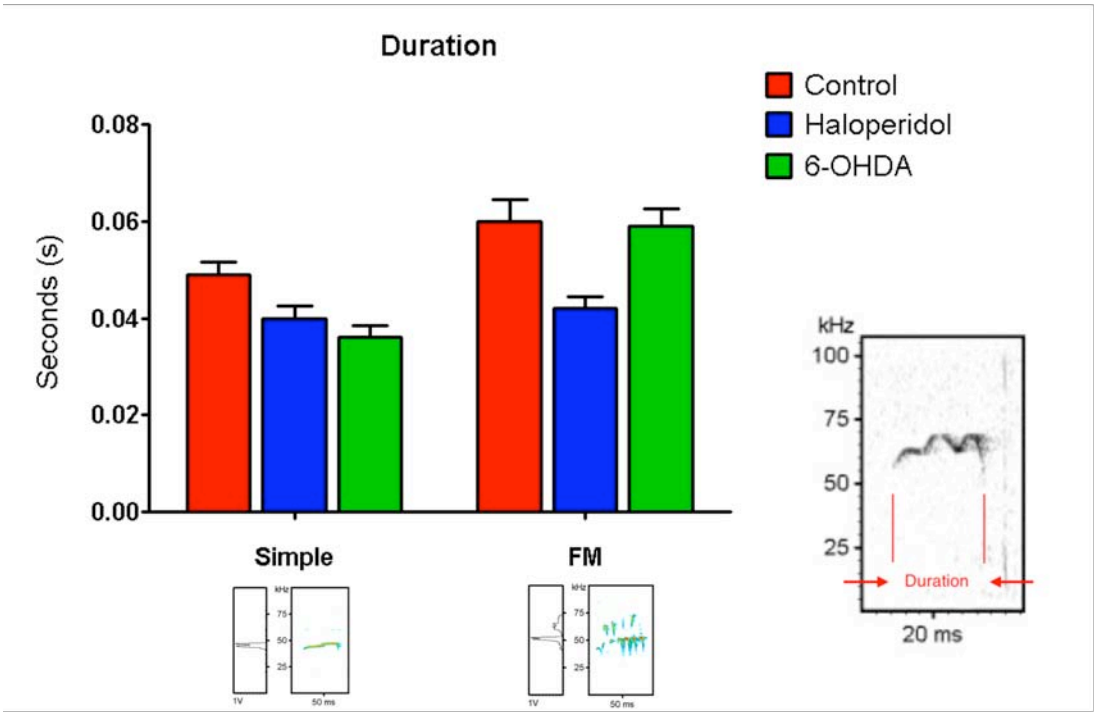

Figure 2.3 Means and standard error of the mean (SEM) for duration measured in seconds (s) in the Control, Haloperidol, and 6-OHDA conditions for the Simple and FM calls.

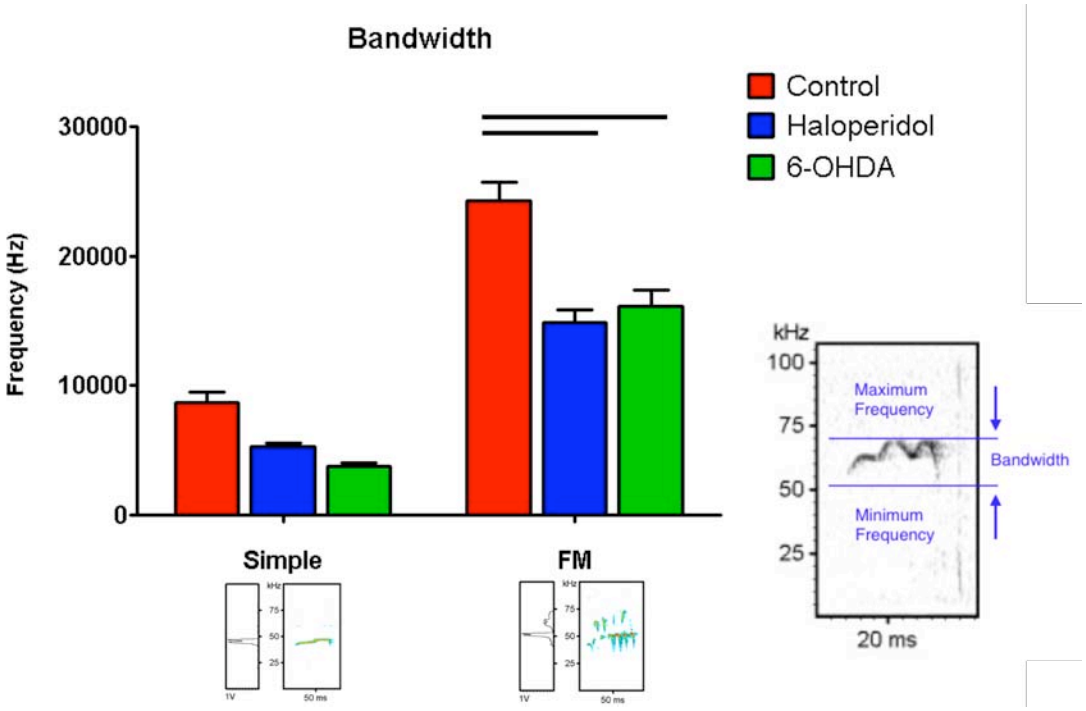

Figure 2.4 Means and SEM for bandwidth, measured in Hertz (Hz), in the Control, Haloperidol, and 6-OHDA conditions for the Simple and FM calls ($p < .01$).

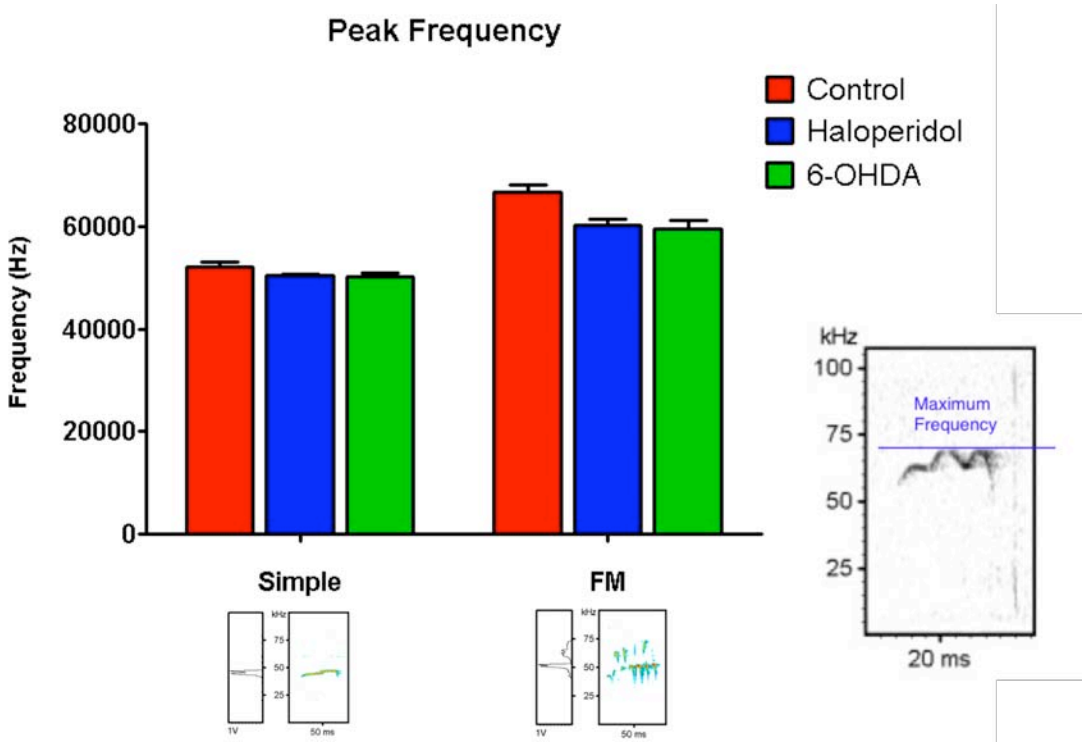

Figure 2.5 Means and SEM of maximum frequency measured in Hertz (Hz) in the Control, Haloperidol, and 6-OHDA conditions for the Simple and FM calls.

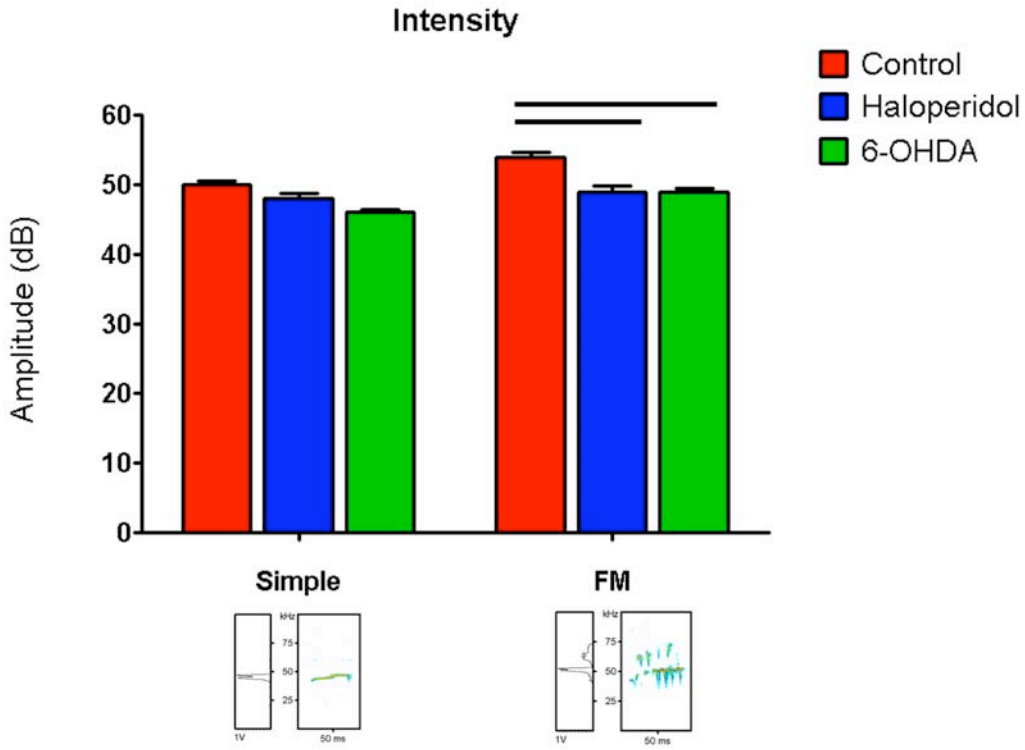

Figure 2.6 Means and SEM for maximum intensity measured in dB in the Control, Haloperidol, and 6-OHDA conditions for the Simple and FM calls. A more negative value represents a call with less intensity ($p < .01$).

Chapter Three: Parkinsonian rat mating calls are unappealing to females

INTRODUCTION

The quality of USVs can be examined in this animal model. In PD patients, voice quality deficit indeed decreased loudness and pitch variation (Darley et al., 1969; Ramig et al., 1996), which can be assessed by rat USVs using playbacks of previously recorded USVs that also lack intensity in volume and variations in pitch (Ciucci, Ma, et al., 2007). In addition, the quality of the calls can be evaluated by a female mate choice test commonly used in frogs and rodents. Preference mate choice tests have been used to indicate the attractiveness of a special trait in animals (Bass and Grober, 2001; Hauser, 2007; Woolley and Doupe, 2008). Female Tungara frogs (Ryan, 1980) are attracted to complex male mating calls compared to a simple call. Thus, they spend more time on the complex call side of an enclosure compared to the simple call side. In rodents there have been experiments conducted to test the function of ultrasonic calls made during sexual interaction in rodents. (Pomerantz et al., 1983; White et al., 1993). Females are more attracted to calls recorded from males that are non-castrated in rodents. In hamsters, a special call elicited from the

male after the female hamster was removed also induced or prolonged the lordodic activity in the receptive female (Cherry, 1989).

The presence of dopamine (DA) in the basal ganglia may be crucial for precise vocalization across species. In Parkinson's disease (PD), speech impairment is common even in the early stages when DA cell loss is not yet severe and movement symptoms are still mainly unilateral (Logemann et al., 1978). In songbirds, DA in Area X (the basal ganglia circuit in birds) modulates singing behavior (Sasaki et al., 2006). In Chapter 2, data were presented showing that in rats, a very low dose of a DA antagonist (haloperidol, a typical antipsychotic drug) or unilateral nigrostriatal DA depletion degrades the quality of 50-kHz range ultrasonic vocalizations (USV) used for mate calling during courtship (see also Ciucci, Ma et al., 2007). USV intensity and pitch variation are reduced, but not the number of calls or the motivation to mate (go to www.schallertlab.org/media to hear human-audible, frequency divided examples of these calls).

Female rats are less likely to mate with de-vocalized male rats than with intact male rats and will readily approach a speaker broadcasting 50-kHz male mating calls and engage in sexual solicitation behaviors, particularly in the presence of urine odors from un-castrated males (Thomas et al., 1982). It has been suggested that sexual behavior in females is enhanced by a combination of male mate calls and olfactory cues. The present study is the first to ask whether auditory information *alone* can be used by female rodents to discriminate between high and low quality

USVs.

METHODS

Animals and housing

26 female Long-Evans rats, obtained from Charles River and aged 4 months were used for playback experiments. All animals were pair-housed in groups of two in polycarbonate cages on a reversed 12:12 hour light: dark cycle. All testing occurred during the dark period of the cycle. Food and water were available *ad libitum*. All experiments conducted were approved by the University of Texas Animal Care and Use Committee.

Sexual Experience

Female rats were randomly selected into sexually experienced (n=16) and sexually naive (n=10) groups. Females were brought into estrous through i.p. injections of 10 μg of estradiol (Sigma, USA) and 500 μg of progesterone (Sigma, USA) at 48 hrs and 4 hrs prior to behavioral testing, respectively. The sexually experienced group was given five opportunities over 15 days during estrus to gain sexual experience with sexually experienced males. Both groups were habituated to a polycarbonate T-maze in 10-min sessions 5 times prior to the experiment.

Acoustic stimuli

Mating calls of control and unilateral DA-depleted male rats for playback were selected from recordings from our previous study (Ciucci, Ma et al., 2007). DA

plays a major role in 50-kHz range USV production, as in other motor behaviors (Ahrens et al., 2009; Burgdorf et al., 2001; Schwarting and Huston, 1996; Thomas et al., 1982; Wintink and Brudzynski, 2001). A 20 second clip of USV mating calls was excerpted from randomly selected, but representative, control and unilateral DA-depleted male recordings. Stimuli were looped and presented continuously up to 2 minutes through a high speed D/A board (PCI-6221, National Instrument, Austin, TX) connected with an ultrasonic speaker (Fountek, China) (diagram shown in Figure 3.1). Speakers broadcasting USVs stimuli from control vs. DA depleted rats were randomized between sessions. Intensity was calibrated to match our previous mating call recording study for both stimuli. Only 50-kHz range calls were present in the sample. Sonograms from both stimuli are shown in Figure 3.2. 1-sec samples of frequency divided audio clips (X20) for human hearing purpose for both stimuli are provided online.

Behavioral testing

Behavioral testing was carried out in low red lighting for all animals. A 5-min period of silence in the T-maze preceded each 2-min playback phase for each female (speaker arms: 40 cm; neutral arm: 36 cm). Behavior was recorded from an infrared camera (Panasonic, Japan). Time spent exploring the left and right sections of the box was assessed during the 2-min test. Data indicated marked female preference for normal male calls. The T-maze was cleaned with 70% alcohol before each test. A representative video of female choosing between speakers is provided

online.

RESULTS & DISCUSSION

A 2-speaker playback system was used. Two groups of estrous female rats - sexually naive and sexually experienced - were primed with estrogen and progesterone and tested in a T-maze. An ultrasonic speaker was mounted on each of the two arms of the T-maze. USV mating calls, recorded from sexually experienced males during exposure to female odors, were broadcasted simultaneously from both speakers inside the maze, as shown in Figure 3.3. One speaker played back USVs of a control male rat and the other played the recorded USVs from a male rat exposed to unilateral nigrostriatal infusion of the DA neurotoxin 6-OHDA, a common animal model for PD. Each two-minute playback session started when the female rat was located in the neutral arm of the T-maze. The investigation time for each speaker was recorded. Results are shown in Figure 3.4. Normal females, both sexually naive and sexually experienced, spent more time investigating the control male's mating call than that of the unilaterally DA depleted rat, indicating a loss of attractiveness in the latter call (naïve: $p = 0.0003$; experienced: $p = 0.0019$). Representative sonograms from normal and parkinsonian model males are shown in Figure 3.5. As shown in Figure 3.6(a), bandwidth, an index of USV quality, was decreased in the hemi-parkinson rats. In addition, striatal DA, measured through high pressure liquid chromatography with electrochemical detection, was severely depleted in the brain

hemisphere where the neurotoxin was infused (Figure 3.6(b)).

Previous studies have shown that rodents rely heavily on chemical communication for mating behavior (Johnston, 2003). We show that USVs are sufficient for female rats to select a "healthy" male over a dopamine-depleted male. This provides researchers a robust behavioral assay for investigating behavioral responses to subtle changes in vocalization as a result of neurochemical imbalance or other mechanistic dysfunction. Additionally, auditory information may provide a key signal for female rats to judge the mate status of males, as has been suggested for some frogs (Ryan, 1980). Female rats also appear to be able to take advantage of relatively distant vocal information to select potential mates, a benefit that may include avoidance of close encounters with less attractive but sexually motivated males. Olfactory cues, in contrast, would not provide with certainty the presence or current location of distant males.

The present findings may contribute to a better understanding of brain mechanisms of phonation and mammalian communication. Moreover, the acoustic playback approach may be helpful in the development of pre-clinical interventions and in the assessment of their potential efficacy for clinical treatments for voice degradation in neurological and psychiatric disorders such as PD, stroke and schizophrenia.

FIGURES

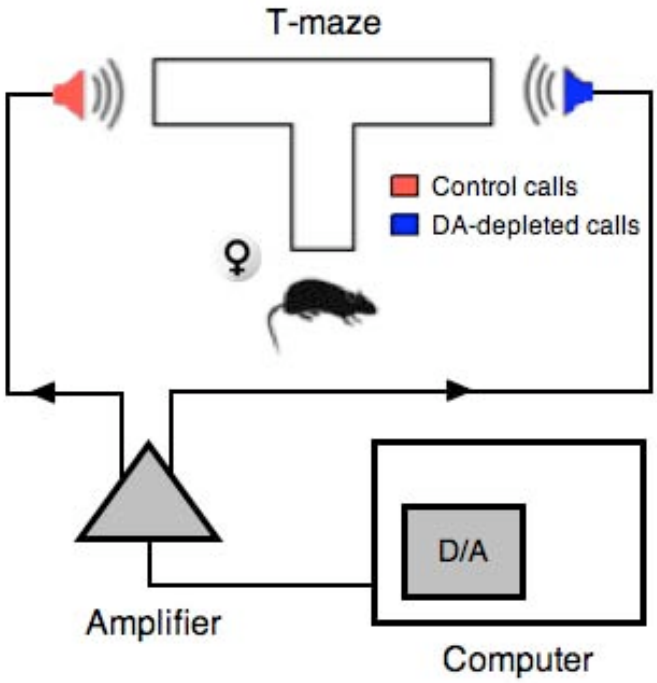

Figure 3.1 Block diagram of T-maze playback system. Recorded male USV mating calls (control, DA-depleted) were amplified and projected to separate USV speakers for playback.

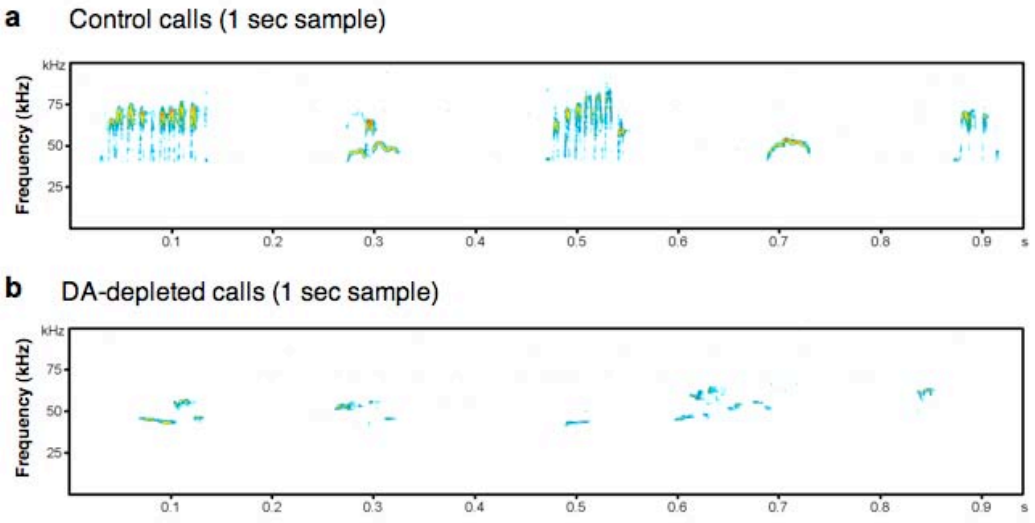

Figure 3.2 Sonogram sample of playback stimuli from control and DA-deplete USVs. (a) 1-sec sample sonogram from recorded control male USV mating call. (b) 1-sec sample sonogram from recorded DA-depleted male USV mating call. Frequency divided versions (X20) of (a)(b) wavefiles are provided online.

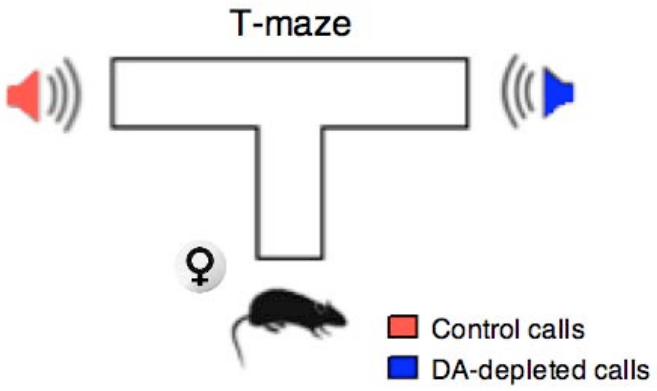

Figure 3.3 T-maze setup for simultaneous playback of male PD and control USVs to estrous females.

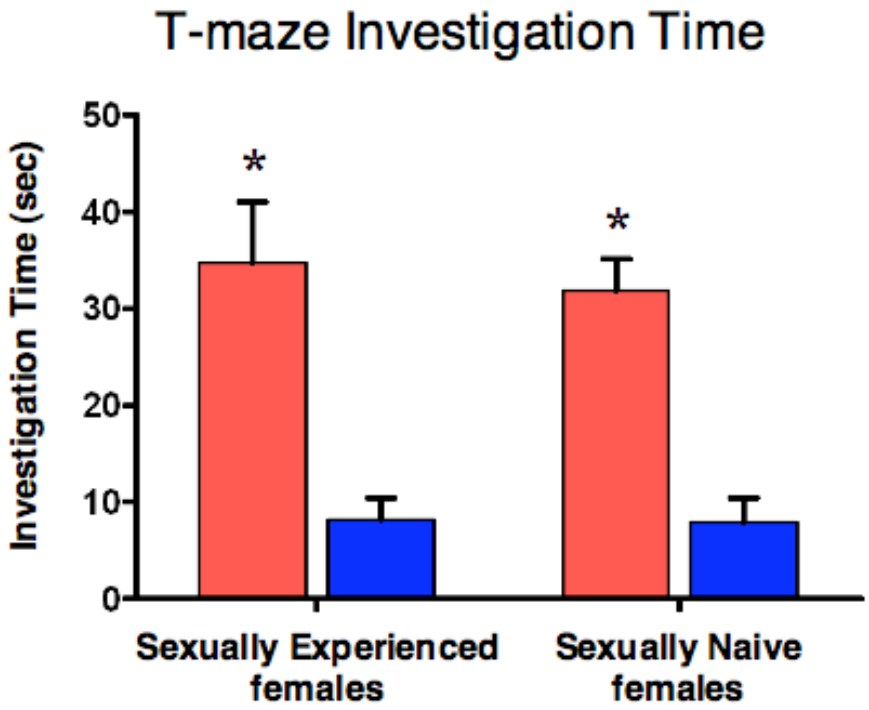

Figure 3.4 Investigation time for the sexually experienced and sexually naïve females. Both groups showed significantly greater interest in the control male calls (naïve: $p = 0.0003$; experienced: $p = 0.0019$).

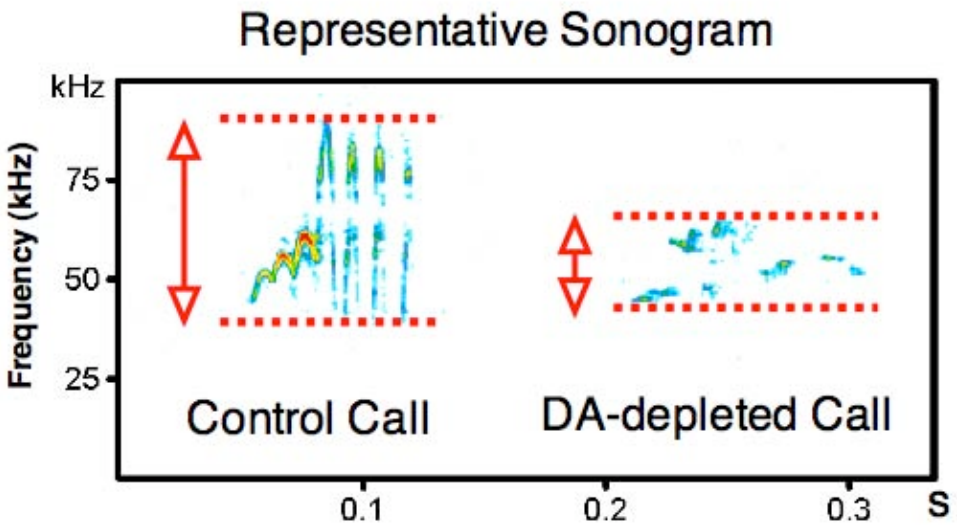

Figure 3.5 Representative sonogram of one of the USV syllables from control and DA-depleted groups. Double arrow line depicts bandwidth obtained from upper and lower frequencies.

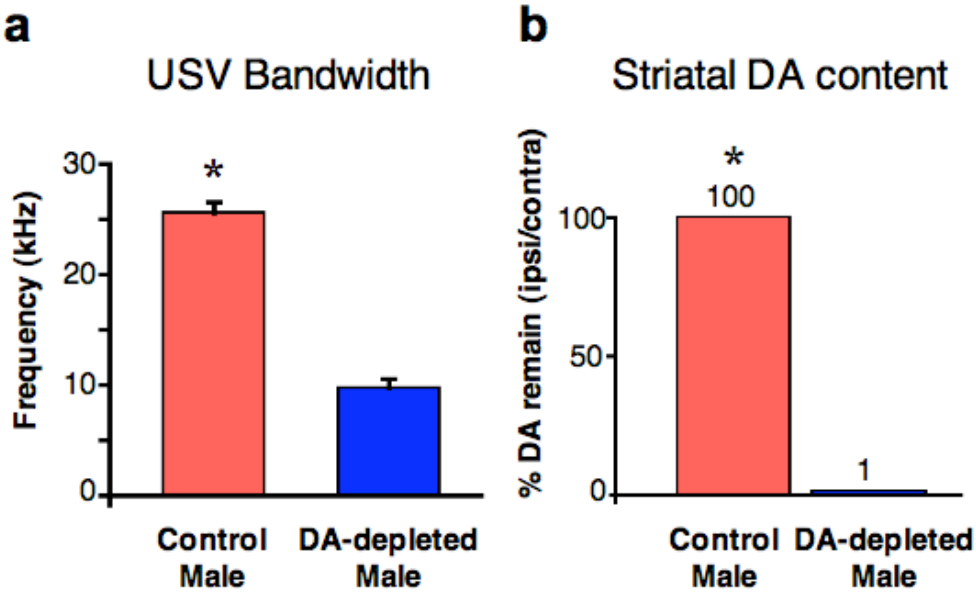

Figure 3.6 USV bandwidth and striatal dopamine content comparison between control and dopamine depleted males. (a) USV bandwidth (lowest to highest frequency) recorded from a hemi-parkinson rat is reliably narrower than that of a control rat. (b) DA was severely depleted in the striatum of the 6-OHDA-treated hemisphere.

Chapter Four: Using bite sounds as a measure to detect biting strength loss in dopamine depleted animals

INTRODUCTION

Parkinson's disease patients commonly exhibit eating and swallowing disorders (Athlin et al., 1989; Bird et al., 1994; Fuh et al., 1997; Miller et al., 2006). The progressive degenerative nature of this disease greatly diminishes the quality of life in patients. Though the hallmark sign of PD is limb motor function loss, including tremor, gait deficit, akinesia, etc., patients also suffer from a degradation of swallowing, mastication, speech production, and respiration, all of which impact their daily functioning.

Animal models of PD are useful for studying the sensorimotor effects of the disease. In laboratory rats, dopamine depletion in the nigro-striatal pathway results in a variety of sensorimotor deficits. The majority of research has focused on deficits in limb motor function (Carter et al., 1992; Ceravolo et al., 2001; Chen et al., 2005; Djaldetti et al., 2006). Recently, there has been increased interest in the subclinical symptoms, such as olfactory loss, digit function impairment, and vocalization degradation (Becker et al., 2002; Sommer et al., 2004; Ciucci, Ma et al., 2007; Koop et al., 2008). While these studies provide insight about the effects of DA loss in the nigro-striatal pathway, little is known about the resulting swallowing and mastication disorder that follows DA depletion. Due to the complexity of the intrinsic muscles and neuronal pathways involved in mastication and swallowing, an effective measure

to quantify the deficit during the eating process is needed.

Previous methods for studying swallowing disorders involve measuring the difference in amount of food before and after the animal ate (Szczypka et al., 1999) or the amount of time the ingestion took place (Kitayama et al., 2007). This requires processing time for the animal to display a quantifiable difference. Other studies of mastication disorders rely on a specially designed apparatus that the animal applies force to (Nies and Ro, 2004; Ro, 2005). This requires extensive preliminary training and appears to be an adverse experience for the animal. Although the studies mentioned provide information from different aspects of swallowing or mastication deficits, none is capable of authentically capturing the deficit during eating.

In this study, we have designed a novel and simple way of quantifying deficits of mastication in Parkinsonian animals when eating a piece of pasta. Whishaw and colleagues have shown that rats with CNS damage display dexterous deficits while eating a long, thin, dry piece of pasta (Whishaw et al., 1998). This pasta eating test has further been explored by Allred et al. (Allred et al., 2008) through the development of a simple quantitative method to describe the loss of digit function in stroke and PD animal models. In the study at hand, we harnessed the use of this pasta eating method in a novel way. Instead of focusing on the digit position and use while the animal eats, we draw our attention to the eating sound pattern while the animal eats the pasta. Through recording and analyzing the eating sound pattern, we are able to non-invasively quantify the deficits related to mastication

strength and swallowing through simple signal processing methods. In unilaterally DA depleted rats, a significant difference was found in the biting pattern and strength compared to control group animals.

METHODS

Unilateral dopamine depletion

Rats were pretreated with 0.1 mg/kg atropine sulfate s.c. (to dry respiratory tract secretions) and 20 mg/kg desipramine i.p. (to protect noradrenergic neurons) 10 min prior to anesthesia. Anesthesia was induced with 40 mg/kg pentobarbital i.p. and maintained throughout surgery with booster injections of 80 mg/kg chloral hydrate i.p. as needed. A small hole was drilled through the skull at 1.5mm lateral to midline and 4.3mm posterior to bregma. A microsyringe needle was slowly lowered through the center of this hole to a point 8.0mm ventral to the dural surface, to target nigrostriatal dopaminergic axons in the medial forebrain bundle. A solution of 8 *u*g (free base weight) of 6-OHDA HBr (a catecholamine neurotoxin; Sigma) dissolved in 2*u*l of artificial cerebrospinal fluid (ACSF) containing 0.05% (w/v) ascorbic acid was infused at a rate of 0.2 *u*l/min. Sham operated controls received infusions of the vehicle (ACSF plus ascorbic acid). At the end of the 10 min infusion the needle was left in place for an additional 2 min before being slowly retracted. The skull hole was sealed with bone wax. These rats were tested approximately 6–7 weeks post-operatively.

Recording pasta biting sound and data analysis

Prior to the experiment, rats were habituated to pieces of pasta 7 cm long in their home cage. Food restriction was implemented for the subject rats the night before recording. A video camera on a tripod stand was placed in front of the home cages for overnight habituation. On experiment day, both video and audio of the pasta ingestion were recorded in the dark through the video camcorder. A total of three sessions of pasta biting from the same rat were recorded. After downloading the recording into the computer, the audio portion of the video for each subject rat was extracted through QuickTime software (Apple, USA). Inter-bite intervals and amplitude from each bite recording were extracted through sound analysis software Saslab Pro (Avisoft, Germany).

X-ray imaging of pasta biting: X-ray cineoradiography

A sham group (N=6) and a 6-OHDA group (N=4) were used in this study. Subject rats were confined in a transparent tube of 4 inch diameter located in the center of the C-arm. Pasta mixed with 80% barium sulfate was custom made locally and clipped into 7 cm long pieces (Pasta & Co., Austin, TX). This enabled us to visualize the pasta bits inside the rat through X-ray imaging. X-ray imaging was conducted through a mobile C-arm unit (Cardiac Digital Mobile Imaging System, USA). A pulsed digital cine (up to 150 mA) at 30 pulses per second with 10-ms pulse width with 1000X1000 imaging resolution was acquired, allowing digital recording at 30 frames per second. The pasta biting sound was recorded with a

directional microphone (Sennheiser, USA) 20 cm from the tube. Digital video signal from the fluoroscope and the acoustic biting signal were recorded by a mini-DV recorder (Sony, USA) for further analysis.

Statistical Analysis

SPSS v15 for Mac OS X was used for all statistical analyse with significance set at $p < 0.05$, and unpaired t test with Welch's correction was applied to determine the p value.

RESULTS

Biting pattern change

Two groups of rats were subjected to the pasta biting test. Both the sham control (n=6) and unilateral 6-OHDA lesioned groups (n=4) had 3 sessions of pasta recording for each rat. From the sonogram of the audio recording, in Figure 4.1, each vertical line represents a physical bite of the pasta from the subject rat. The biting pattern in the sham control group had consistent, firm bites compared to the 6-OHDA group. We observed an overall biting pattern change between the two groups due to the dopamine depletion in the nigra-striatal pathway. This inconsistency was further quantified by measuring the time between each bite from the sonogram, i.e. the inter-bite intervals, from the two groups. In Figure 4.2, we found a significant increase in time of the inter-bite interval for the 6-OHDA group relative to that of the

sham control group ($t(20)=2.521$, $p=0.02$).

Biting strength loss

The biting strength loss seen in PD patients was also modeled in our pasta biting test. By measuring the intensity of the biting spikes in our audio recordings, a significant decrease in the intensity for the 6-OHDA group compared to the sham control group was found ($t(24)=2.765$, $p=0.01$), as shown in Figure 4.3. The representation of pasta biting sound intensity positively correlated to the physical biting strength. A decrease in the sound intensity represents a loss in the subject's biting strength. This sound intensity/biting strength loss seen in the 6-OHDA group is also significantly correlated with the results of the cylinder behavioral test ($R^2=0.3816$, $p=0.006$), as shown in Figure 4.4.

Inefficient bites revealed in X-ray cineoradiography

With X-ray cineoradiography, further biting patterns inside the subject's mouth was revealed through contrasted barium sulfate pasta. A consecutive biting sequence resulting in individual pasta bits after each bite was observed in control rats. In contrast, the 6-OHDA rats were not as successful. Bites in the 6-OHDA group often resulted in "taps" on the pasta, rather than actual bites, and these were not detected in the sonogram. In Figure 4.5, the 6-OHDA group suffered from a significant loss in the efficiency of biting compared to the control group.

Increased pasta bit size in 6-OHDA group

The size of the pasta bits gnawed off from subject rats was also revealed through X-ray cineoradiography. In Figure 4.6(a)(b), image analysis was applied on the pasta bits from representative rats from both groups. Mean pasta bit size was significantly larger in the 6-OHDA group compared to the control group ($t(25)=5.825$, $p<0.0001$), shown in Figure 4.7. These results align with the long inter-bite interval results as well as the low successful biting rate from the inefficient biting. The pasta bit size was reversely correlated with the successful bite rate in the 6-OHDA group.

DISCUSSION

Studies have shown that early intervention, such as exercise, can help prolong the quality of life in later stages of PD (Comella et al., 1994; Chen et al., 2005). However, early intervention requires early identification of symptoms, leading to early diagnosis of the disease. Oromotor deficits, including speech and swallowing disorders is prevalent amongst 80% of PD patients and often present during early stages of the disease (Logemann et al., 1978). Currently, there is no beneficial animal model for detecting oromotor deficits in PD, which limits the possibility of early detection.

Swallowing disorders have been reported more commonly in Parkinson's disease than mastication loss (Athlin et al., 1989; Fuh et al., 1997; Potulska et al., 2003; Miller et al., 2006; Sapir et al., 2008). In this original study, I have developed

a novel method to measure mastication impairment due to the loss of dopamine neurons in unilaterally lesioned Parkinsonian rodents. The positive correlation between the sound of pasta biting and the actual bite strength has aided us in developing a non-invasive and easy to administer animal model for oromotor deficits. Through analyzing the sound of pasta bites between control and 6-OHDA groups, a significant reduction of intensity was observed in the 6-OHDA group. Though it could be argued that the observed biting pattern change may be due in part to a handling deficit of the animal's front paw (Whishaw et al., 1998), the intensity loss in the 6-OHDA rats, is likely to be unaffected by pasta handling ability. A direct measurement of bite force from dopamine depleted animals using force transducer should be carried out in a future study. Nevertheless, the sound intensity measurement from the pasta biting test appears to be a reliable and repeatable index of mechanistic strength in oromotor mastication function.

In addition to intensity loss, biting patterns from the sonograms were different between control and dopamine depleted animals. The overall inter-bite interval was significantly increased in the 6-OHDA group compared to the control group. This result aligns with other studies showing increased timing of food in-take for Parkinsonian animals (Kitayama et al., 2007; Allred et al., 2008) as individual bits of pasta took longer to bite off, resulting in a longer ingestion period. Many factors might contribute to this pattern of change. One of these factors could involve the animal's handling instability of the pasta. As Allred et al. have shown, dopamine

depleted animals lose digit functions in the contralateral limb during pasta handling. Another factor could also involve strength loss in the tongue or its sensorimotor integration that is required for precise oromotor control during food ingestion (Whishaw et al., 1997). One other possibility could be that vacuous jaw movements, observed by Salamone et al. (Jicha and Salamone, 1991) could influence biting capacity. These movements are thought to resemble tremor in PD patients, which is only occasionally seen in Parkinsonian rat (Cenci et al., 2002).

Our investigation with X-ray cineradiography has further assisted us in understanding the deficit occuring inside the animal's mouth. The results provide major evidence that inefficient bites are present in the 6-OHDA group. Compared to a control animal's biting behavior, in which a solid bite resulted in a broken piece of pasta bit, the dopamine depleted animals typically made several inefficient attempts of biting in-between two solid bites. When reviewed from the X-ray movies, the animal consistently seemed to tap weakly on the pasta instead of biting it off. These data support our observation of the prolonged inter-bite interval 6-OHDA animals exhibit during the sound biting test. This inefficient biting appears also to explain why 6-OHDA animals have larger pasta bits (under X-ray) when compared to the control animals.

In general, compared to other measurements in mastication studies, the pasta biting test is extremely simple to administer. It is also non-invasive for the animal and makes it easy to collect and analyze data. As early detection increasingly is

being more emphasized in neurodegenerative diseases, such as Parkinson's disease, ALS, or even stroke, and given the possibility that oromotor deficits might precede deficits in other motor systems, developing an animal model test that might benefit the field seems crucial for the study of early intervention. Analysis of bite sounds in future translational studies should not be limited to laboratory rodents.

FIGURES

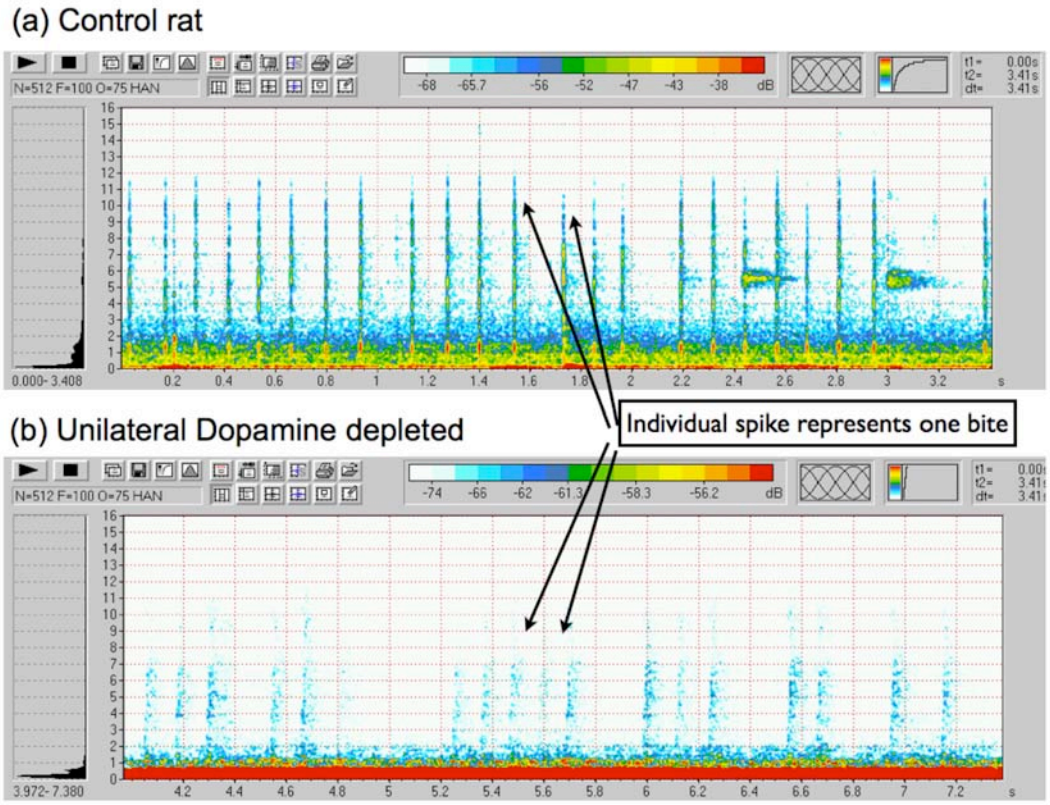

Figure 4.1 Representative sonograms of pasta bite from (a) control rat, (b) 6-OHDA unilateral dopamine depleted rat. Each spike represents a wide-band signal produced by a bite from the pasta. X-axis is time and Y-axis depicts frequency. Color represents the intensity of the signal, which the color red has the highest intensity relative to other colors.

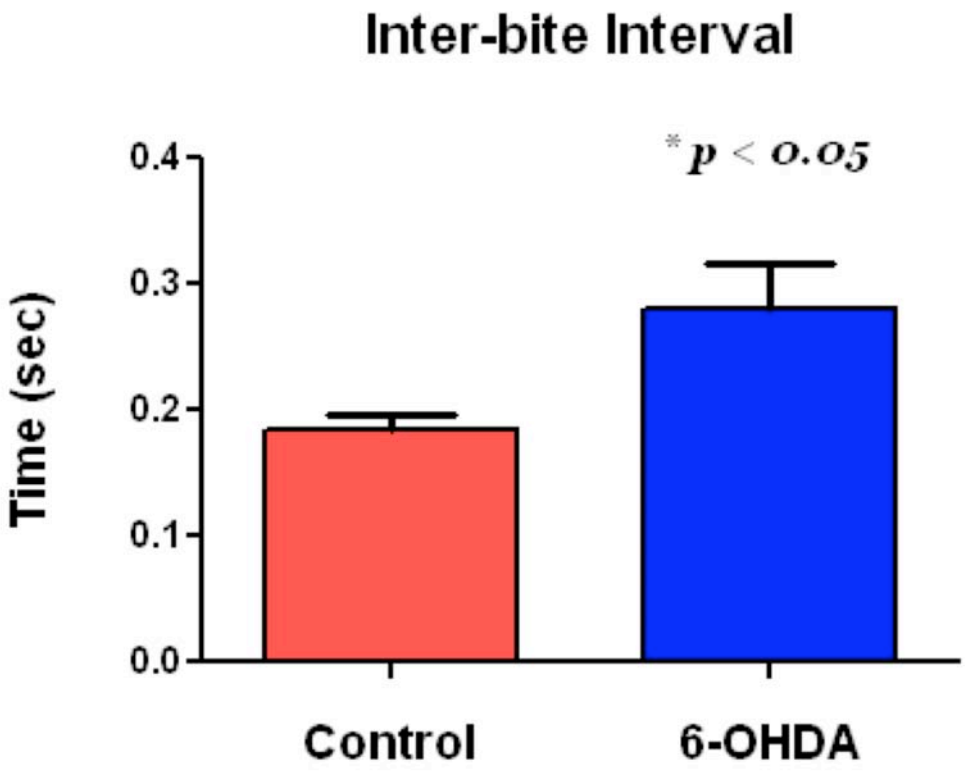

Figure 4.2 Inter-bite interval of pasta biting between control and 6-OHDA groups. Unilateral dopamine depleted rats suffer from a prolonged inter-bite interval compared to the control rats.

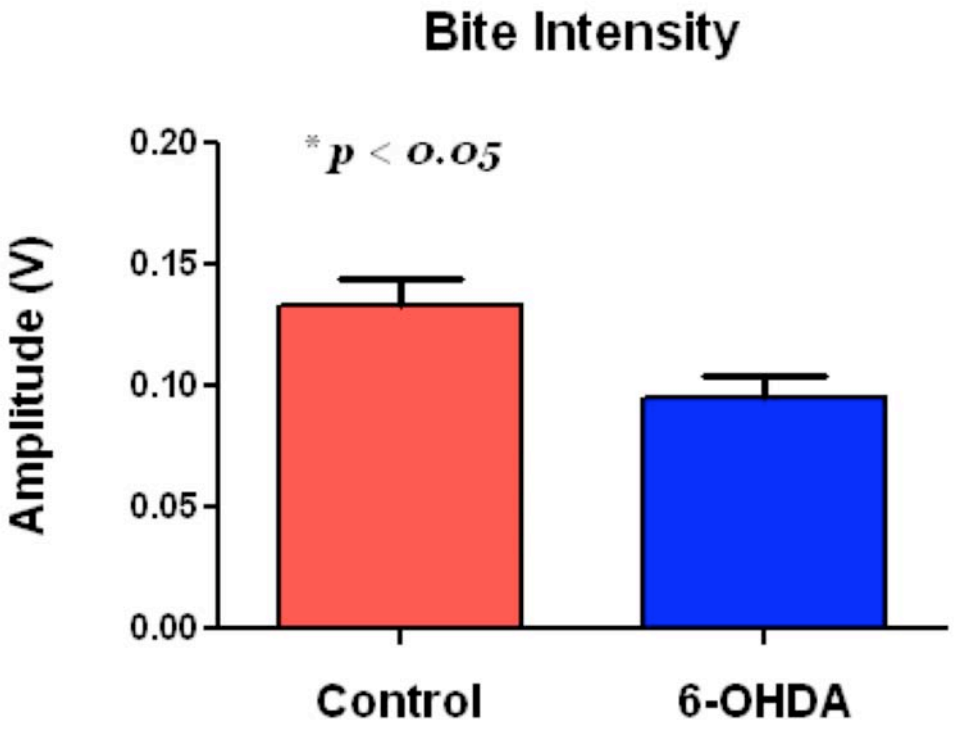

Figure 4.3 Bite intensity of pasta biting between control and 6-OHDA groups. Unilateral dopamine depleted rats suffer a significant loss of biting strength compared to the control rats.

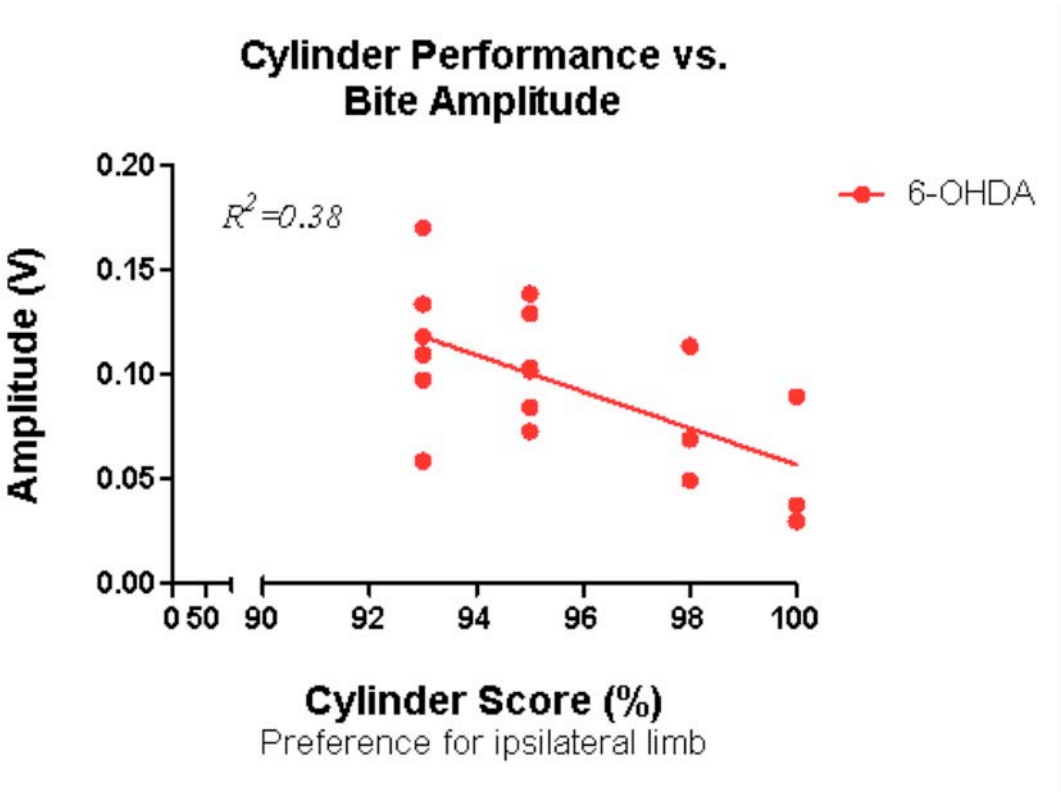

Figure 4.4 R square correlation between Bite Intensity and Schallert Cylinder Score in the 6-OHDA dopamine depleted rats.

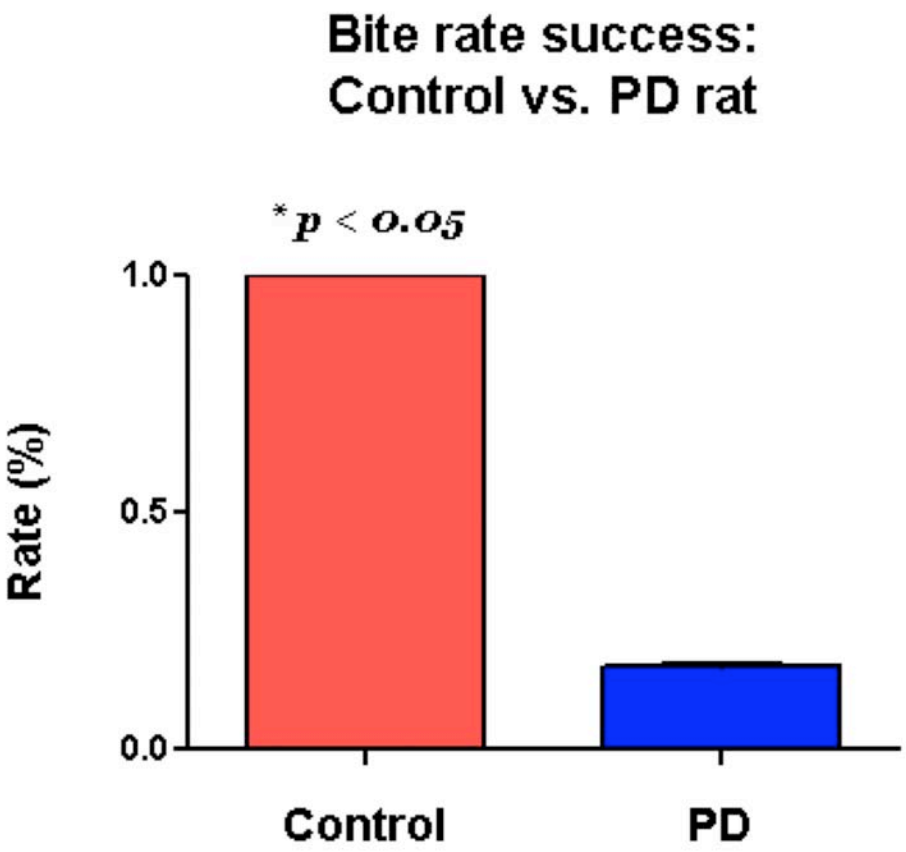

Figure 4.5 In-efficient biting in the 6-OHDA group compared to the control group. "Taps" were observed in between actual bites in the unilateral dopamine depleted group, compared to the firm bites to bits in the control group.

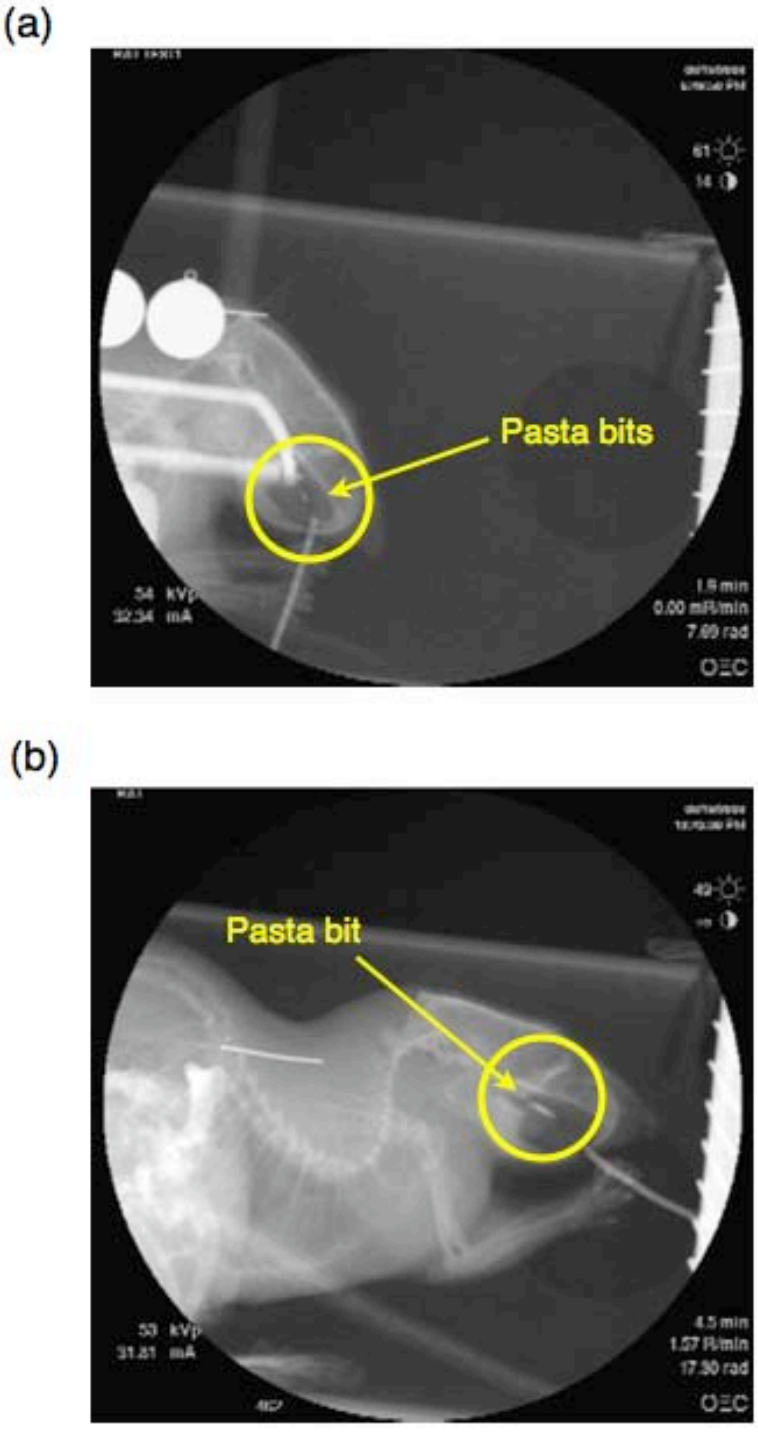

Figure 4.6 Representing X-ray images comparing pasta bit size from (a) control rat vs. (b) 6-OHDA rat. Bit size is significantly larger in the 6-OHDA rat.

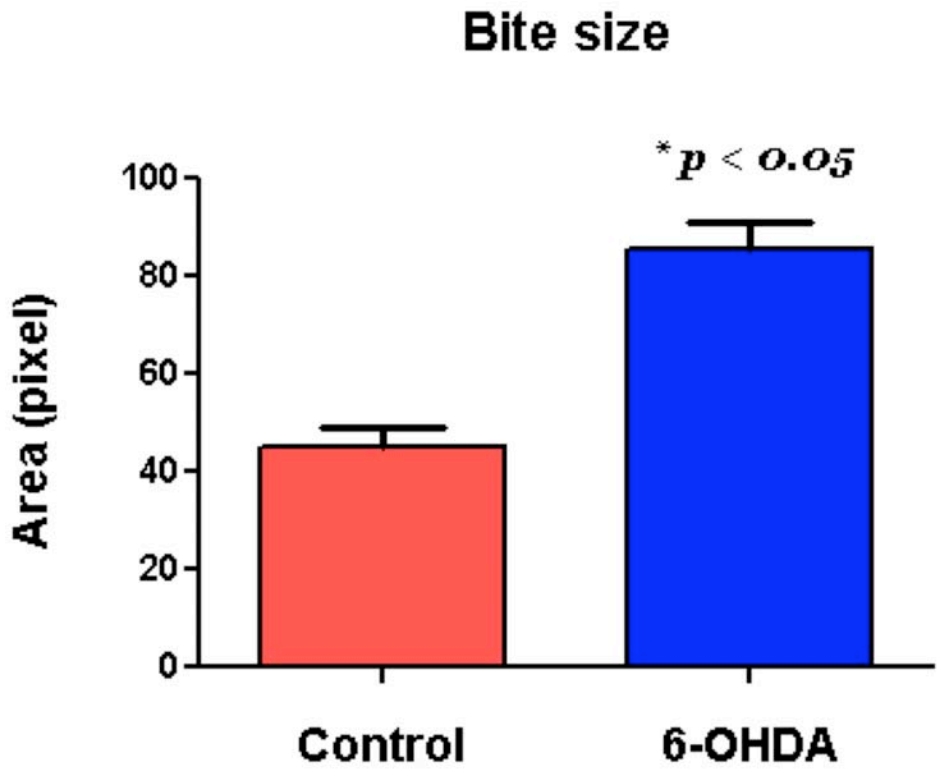

Figure 4.7 Individual pasta bit size under X-ray cineradiography between control and 6-OHDA groups. Pasta bits were analyzed by ImageJ.

General Discussion

Rodent ultrasound vocalization (USV) is a subtle yet complex motor output. It requires fine sensorimotor coordination to produce precise vocal projection in acoustic space compared to other behavioral functions. Due to its intrinsic features, including above sonic frequency range and very low level of intensity, special techniques are required to authentically capture these whispers in the dark. As more studies involve rodents as a model for language and communication (Lai et al., 2001; Shu et al., 2005; Wohr and Schwarting, 2007; Fujita et al., 2008), communication disorders (Ciucci, Ma et al., 2007), motor degeneration (Miana-Mena et al., 2005; Wooley et al., 2005; Martin et al., 2007), autism disorders (Moy et al., 2006; Jamain et al., 2008), and drug addiction (Ahrens et al., 2009; Mu et al., 2009), the importance of grasping the full potential of USV in laboratory rodents has become necessary. In most studies that involve measuring USV as a behavioral marker, the quantity of USVs emitted is the main measurement reported. The recording devices used are typically bat detectors that tune only to a specific frequency range. Limited by a narrow bandwidth window, only certain flat USV calls that fall into this range will be detected and reported in a frequency divided manner. Additional artifacts, including wide band noise, the animal's locomotion, that fall into this window, will

also trigger the detector and maybe reported as USVs. The USV recording techniques used in this dissertation are based on high speed recording cards and ultrasonic microphones assembled by the author. This system authentically captures the complete spectrum of information from the USVs emitted. Information is then represented in 2-D sonograms for parameter extraction and false report rejection.

To study the ultrasonic vocalizations of laboratory rats, a need for a consistent method to evoke vocalizations from rats is crucial. In Chapter 1, I have developed a method from a natural social context, the rat's courtship behavior, to induce a reliable emission of USVs from male rats. Rats are social animals. They interact and communicate in social groups. Male rats, upon encountering an estrous female, will emit song-like USVs (Holy and Guo, 2005) during the courtship behavior. Previous studies have suggested USV mating calls from males can trigger receptive (solicitation) behavior of the female rat. This includes darting and ear wiggling from females (Barfield et al., 1979). From the special design of the recording chamber, it is ensured that a consistent flow of USVs, emitted only from the male and not the female, is recorded and used as a basis to study the qualitative changes in vocalization.

In Chapter 2, with the loss of dopamine in the nigra-striatal pathway, courtship calls from male rodents degrade in intensity and lose the high pitch variation as seen

in the 50-Khz calls. It is interesting, though, that with only a unilateral insult to the DA cells of the nigra-striatal pathway was sufficient to cause a significant degree of deficit in the rat's vocalization. This result takes into account that muscles recruited in the production of vocalization are bilaterally innervated from the CNS in contrast to the largely lateralized connections for limb function. Vocalization seems to be very sensitive to any perturbation occurring in the responsible muscles, neurons or even neurotransmitters. However, one might argue the deficit seen in the USV of dopamine depleted male rat might result in the motivational state of the rat, especially when the lesion was conducted at the medial forebrain bundle (MFB). Because the dopamine loss at the MFB, the subject rat might have less motivation to call when encountering the female odor in the recording chamber, thus resulting a qualitative degradation in their vocalization. This hypothesis was negated by the mounting time measured when the subject male encountered an estrous female during sexual interaction prior to entering the USV recording chamber. Male rats from 3 groups (control, Haloperidol, and 6-OHDA) all mounted the females within adequate timing with no significant difference. This clearly indicates this natural stimulus, the estrous female, is still potent for the males to initiate mounting behavior. This further helped us tease out the dichotomy between motivation versus motor when studying the deficit in the rat's vocalization.

It is also interesting how this disconcert in vocalization creates a bias in the female rats, which are the ones perceiving the mating calls, to spend more time in front of the normal mating calls compared to the PD ones. This opens possibilities of testing target oriented vocalization training for PD animals to study the efficacy of vocalization therapy, and the combination with Levodopa treatment, as the drug itself shows no significant recovery in vocalization during pilot study. The results from playback experiments also have the potential to expand into auditory neuroethology studies in rodents: acoustic information alone, without the presence of olfactory information, is potent enough for animals to make crucial decisions regarding their reproduction success.

Despite the fact that 50-kHz calls are commonly associated with positive affective states, the role of 50-kHz calls still needs further investigation. This is especially the case for the 2 major categories within 50-kHz range: the flat calls versus the complex calls (FM and Harmonics). Although both of these call types share the same frequency energy at 50kHz, we see a gradual proportional change in the production of more complex calls to more flat calls when comparing the normal control rats, the dopamine-antagonist (Haloperidol) treated rats, and the 6-OHDA unilateral dopamine depleted rats. Could this ratio be indicating the rewarding effect the animal receives in a mating context? Could the depletion of dopamine in the

nigrostriatal pathway result in a downgrade effect in reward quality that resulted in more production of the flat calls? Will the female rats in a playback environment prefer the complex calls to the flat calls? What is the function of the flat calls? And what about the complex calls? Are they just a mere signal to attract conspecifics to come for a variety of reinforcement, not just sexual activity? Although not many studies have investigated this topic, if we look at the above questions from a drug addiction perspective, there may be some insights that can inspire us. In a complimentary study, we increased the level of dopamine through the intravenous infusion of a psychostimulant drug, amphetamine. Ahrens et al. (Ahrens et al., 2009) found a sensitization effect on the numbers of vocalized 50-kHz USVs after intravenous injections of amphetamine. Specifically, this influx of dopamine in the CNS caused the rats to vocalize more of the complex calls after repeated days of drug treatment. This sensitization persisted long after drug discontinuation. The numbers of flat calls, however, were not sensitized. Thus, whereas DA depletion increased the ratio of flat to FM calls, an indirect DA agonist decreased this ratio.

In this dissertation, I also developed a non-invasive biting test for animals that is easy to administer and solely relies on the sound bites from a known food texture. Using the correlation of the sound bite intensity to the force exerted, one is able to measure the incision force from an animal without undergoing complex behavior

training. This biting test also has the potential to be developed into a bite strength therapy for animals by increasing the diameter of the pasta incrementally.

Anatomical evidence for the explanation of degradation in vocalization along side with deficits in biting due to the dopamine depletion in the nigro-striatal pathway can be summarized in Figure G.1. There are 2 major pathways that participate in the output of mammalian vocalization (Jurgen 2009;). The first pathway is a direct pathway that originates from the cortex and passes through the pons that reaches the nucleas ambiguous (NA) in medullary reticular formation (MRF). This is considered the cortical-bulbar pathway that controls the voluntary control of vocalization. The indirect pathway, which originates in the prelimbic region of the medial frontal cortex (PL), further projects down to the lateral hypothalamic area (LH) and interact with the midbrain region, involving the midbrain reticular formation (MRF) and the central gray-rostral part (CGr). The MRF then further projects down to the pons and innervates on the trigeminal motor nucleus of the cranial nerve V (M-V), which is related to the control of biting. The projections from the MRF further interact with the pontine reticular formation (PRF), which projects to the hypoglossal nucleus of cranial nerve XII (XII) that is responsible for the control of the tongue. In the midbrain region, the CGr innervates downward upon the NA for the control of vocalization in mammals. The indirect

pathways for vocalization and oromotor controls during ingestion mentioned above are considered to be innate and are influenced heavily through emotional and motivational aspect. Therefore, it is possible with the lesion in the dopaminergic pathway of the medial forebrain bundle (MFB) that LH area was also affected, resulting in a degradation in both vocalization and oromotor functions in the animal.

Overall, oromotor deficits in PD, including vocalization and mastication strength, are subtle compared to sensorimotor deficits in limb functions. Through this dissertation, several innovative biomarkers, involving acoustic sound made by the animal through encountering an opposite sex or biting from a piece of pasta, have been demonstrated to effectively detect subtle changes in the oromotor function. Considering the complex muscle groups and convoluted nerve innervation within this region (Duffy, 2000), the biomarkers mentioned might provide suitable targets for preclinical exploration of the progression of this neurodegenerative disease.

Figures

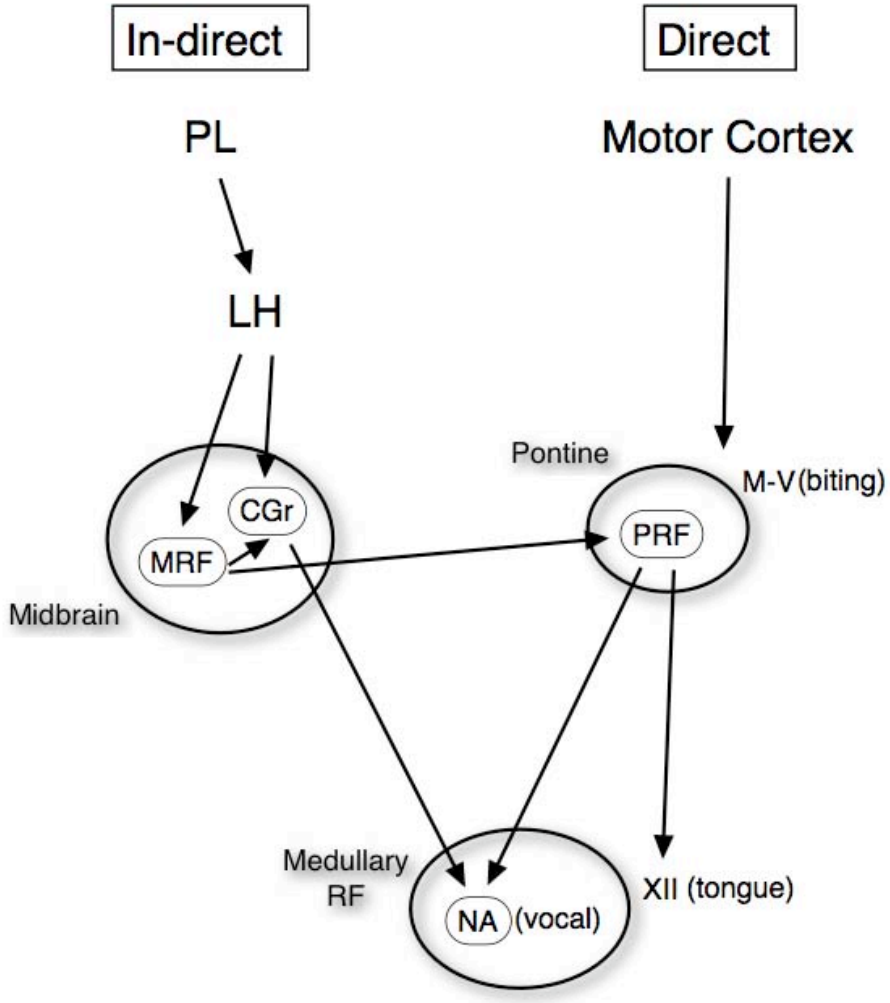

Figure G.1 Direct and indirect motor pathways involving vocalization, tongue movements, and biting functions.

Glossary

6-OHDA	6-hydroxydopamine, a catecholamine neurotoxin
ANOVA	analysis of variance
DA	dopamine
E	estrogen
HPLC	high-pressure liquid chromatography
L-DOPA	levodopa, a drug used in the treatment of Parkinson's
MFB	medial forebrain bundle
PD	Parkinson's disease
P	progesterone
SEM	standard error of the mean
USV	ultrasonic vocalization

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